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(57) Abstract

This invention relates to peptides and peptidomimetic compounds that modulate the function of G-protein-coupled receptors by affecting the ratio of receptor monomer to homo-oligomeric forms. Novel short peptides of a preferred length of up to about 15-20 amino acid residues are modeled on transmembrane domains of G-protein-coupled receptors, whose activities are affected by the formation of oligomers. These novel peptides and peptidomimetic compounds can be used to selectively affect the activity of G-protein-coupling receptors, thereby functioning as potential therapeutic drugs, etc.. A preferred peptide is GIIMGTFTLCWLPFFIVNIV.

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PEPTIDES AND PEPTIDOMIMETIC COMPOUNDS AFFECTING THE ACTIVITY OF G-PROTEIN-COUPLED RECEPTORS BY ALTERING RECEPTOR OLIGOMERIZATION

FIELD OF THE INVENTION

The present invention relates to novel peptides and/or peptidomimetic compounds, methods for generating such compounds, and the use of such compounds to affect receptor oligomerization of G protein-coupled receptors that form multimeric associations for activity.

BACKGROUND OF THE INVENTION

The class of receptors known as G protein-linked receptors (GPCRs) are typically characterized by a 7-helix organization, whereby the receptor protein is believed to traverse the membrane seven times. GPCRs share a common signalling mechanism, whereby signal transduction across the membrane involves intracellular transducer elements known as G proteins. When a chemical messenger binds to a specific site on the extracellular surface of the receptor, the conformation of the receptor changes so that it can interact with and activate a G protein. This causes a molecule, guanosine diphosphate (GDP), that is bound to the surface of the G protein, to be replaced by another molecule, guanosine triphosphate (GTP), triggering another conformational change in the G protein. When GTP is bound to its surface, the G protein regulates the activity of an effector. These effectors include enzymes such as adenylyl cyclase and phospholipase C, channels that are specific for calcium ions (Ca^{2+}), potassium ions (K^+), or sodium ions (Na^+) and certain transport proteins.

In general, activation of GPCRs by transmitters will induce one or another of the following effector responses: activation of adenylyl cyclase, inhibition of adenylyl cyclase or stimulation of phospholipase C activity. When the effector adenylyl cyclase is either activated or inhibited it produces changes in the concentration of the molecule cyclic adenosine monophosphate (cAMP). Another effector, phospholipase C, causes one molecule of phosphatidylinositol-

bisphosphate (PIP_2) to be cleaved into one molecule each of inositol triphosphate (IP_3) and diacylglycerol (DAG); IP_3 then causes calcium ions (Ca^{2+}) to be released into the cytoplasm. Alterations in cellular levels of cAMP and Ca^{2+} are two of the most important intracellular messages that in turn act to alter the behaviour of other target proteins in the cell.

GPCRs may be classified according to the type of signalling pathway they activate in cells. This occurs at the level of the G proteins, which detect and direct signals from diverse receptors to the appropriate effector-response pathway. The three main groups of G proteins are: Gs-like, which mediate adenylyl cyclase activation; Gi-like, which mediate inhibition of adenylyl cyclase; and Gq-like, which mediate activation of phosphoplipase C. Since one receptor can activate many G proteins, the signal can be greatly amplified through this signal transduction pathway.

A wide variety of chemical messengers involved in regulating key functions in the body act through GPCRs. These include neurotransmitters such as dopamine, acetylcholine and serotonin, hormones of the endocrine system such as somatostatin, glucagon and adrenocorticotropin, lipid mediators such as prostaglandins and leukotrienes and immunomodulatory proteins such as interleukin-8 and monocyte-chemoattractant polypeptide. The family of GPCRs also includes the receptors for light (rhodopsin), for odors (olfactory receptors) and for taste (gustatory receptors). Over one hundred different G protein-coupled receptors have been identified in humans, and many more are expected to be discovered. All or most of these receptors are believed to utilize one of the three principal G protein-effector signalling pathways (stimulation or inhibition of adenylyl cyclase or activation of phospholipase C).

Examples of G Protein-Coupled Neurotransmitter Receptors

Inhibits AC	Stimulates AC	Stimulates PLC	Neurotransmitter
m_2, m_4,		m_1, m_3, m_5	Acetylcholine
A_1, A_2			Adenosine
CRF-R			Corticotropin-Releasing Factor
R^c			Cannabinoids
D_2	D_1, D_5		Dopamine
	H_2		Histamine
Y_1, Y_2, Y_3			Neuropeptide Y
β_2-AR	β_2-AR β_1-AR		Norepinephrine. epinephrine
μ, δ, κ	κ		Opioids
5-HT_{1A}, 5-HT_{1B}	5-HT_4	5-HT_2	Serotonin
5-HT_{1D}			

GPCRs are believed to function as monomeric units. Diagrams and text describe the majority of members within this class of receptors as singular units, generally spanning the membrane seven membrane times (For eg. see: descriptions of the muscarinic receptor in *Basic Neurochemistry*, fifth edition, eds: Siegel et al; Raven Press: N.Y.;254-259, 1994). However, it must be noted that there are some GPCRs that are not members of the seven membrane spanning family. For example, the receptor for insulin-like growth factor II that directly activates G_{i-2}, has only a single membrane spanning domain.

In spite of the fact that higher molecular weight species have been previously observed for several GPCRs, these species have largely been referred to as non-specific aggregates (Vasudevan, S., et al., *Eur. J. Biochem.* 227:466-475, 1995). Several studies, including photoaffinity labelling (Avissar, S., et al., *Proc. Natl. Acad. Sci. USA*, 80:156-159, 1983), radiation inactivation (Venter, J.C. and Fraser,

C.M., *Trends Pharmacol. Sci.*, 4:256-258, 1983), cross-linking (Herberg, J.T., et al., *J. Biol. Chem.*, 259:9285-9294, 1985), and hydrodynamic analyses (Peterson, G.L., et al., *Biochemistry* 25:3189-3202, 1986) have produced evidence suggesting cooperativity in binding of agonists to GPCRs, suggesting that they may be part of an oligomeric array (Avissar, S., et al., *supra*). However, a clear physical demonstration that GPCRs can indeed interact as dimers and that such interactions may have functional consequences has not been reported.

It is important to note the distinction between observations that higher molecular weight species (representing oligomeric arrays) have been observed and demonstration that these associations actually play a role in functional activity: the latter being considerably more difficult to substantiate and quantitate than the former. Yet, essential to the observation of enzyme regulation by reversible dissociation is the observation that different molecular weight species have different rates of catalytic activity.

The human β_2 adrenergic receptor has been used as a model to illustrate the common structural features shared by members of the GPCR family (Kobilka, G., *Annu Rev. Neurosci.*, 15:87-114, 1992). Evidence from biochemical (Dohlman et al., *J. Biol. Chem.*, 262:14282-14288, 1987) and immunologic studies of the topology of the β_2 adrenergic receptor (Wang et al. *J. Biol. Chem*, 264: 14424-14431, 1989) supports the model that most GPCRs comprise seven membrane spanning domains.

In view of the diverse functions of GPCRs in the human body, it is not surprising that the pharmaceutical sector has great interest in the development of new drugs which target GPCRs for potential therapeutic applications in a wide range of human pathologies, including psychiatric disorders (depression, psychoses, bipolar disorder), metabolic disorders (diabetes, obesity, anorexia nervosa), cancer, autoimmune disorders, cardiovascular disorders, neurodegenerative disorders (eg. Alzheimer's disease) and pain disorders.

Such drugs may be classified into two types: 1) agonists, which mimic the action of natural

transmitter by provoking activation of G protein-effector signalling pathways when they bind to the transmitter site; and 2) competitive antagonists, which block the binding of the transmitter by occupying the transmitter binding site but do not themselves activate G protein-effector pathways. A useful analogy is that of a lock and key, whereby agonists are different keys which are able to open the same receptor lock, whereas antagonists will block the key-hole but will not open the lock. In a more general view, compounds which can bind to a specific region of the receptor are called ligands: agonists and antagonists are ligands which bind to the transmitter recognition site on the receptor.

Analysis of the effects of ligands on the ability of G protein-coupled receptors to activate signalling pathways has suggested that the receptors exist in two forms or conformations, an 'inactive' conformation which is silent and an 'active' conformation which triggers G protein activation and effector signalling (Gilman, A.G., 1987, *Annu. Rev, Biochem.* 56: 615-649; Levitski, A., 1988, *Science* 241:800-806). Generally, ligands that can cause the receptor to assume the active conformation turn on signalling and are thus agonists. These compounds, at maximally effective concentrations, can elicit a full or partial response, and are termed full and partial agonists, respectively. Ligands that block or otherwise interfere with the interaction of agonists with the receptor, and thereby prevent agonists from activating the receptor, are known as competitive antagonists. These compounds are generally thought to act by binding to the transmitter site, but to possess no intrinsic activity themselves (*i.e.* they do not turn on the signalling function of the receptor.) Studies have shown that competitive antagonists can be further categorized into two classes, 'neutral antagonists' which block agonist binding but have no effect on signalling, and 'inverse agonists' (also known as negative antagonists) which can inhibit the 'background' or basal level of signalling displayed by receptors in the absence of agonists.

This evidence has led to a model in which active and inactive receptors co-exist in the cell in equilibrium, with agonists pushing the equilibrium to the active form, inverse agonists pushing it to the inactive form, and neutral antagonists blocking the chemical messenger site while not favoring either conformation.

The background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Moreover, publications referred to in the following discussion are hereby incorporated by reference in their entireties in this application.

SUMMARY OF THE INVENTION

The present invention resides in the discovery that novel peptide compounds modelled on the transmembrane region of GPCRs can selectively modulate the function of such receptors by affecting the ratio of receptor monomer to multimeric forms (homo-meric or hetero-meric). Specifically, the present invention relates to novel short peptides of a preferred length of up to about 15 - 20 amino acid residues, or peptidomimetic compounds, modeled on transmembrane domains of GPCRs which form oligomers (eg. dimers) for activity that can be used to selectively affect activities of GPCRs. A working example is provided, based on residues 276 - 296 of the β_2-adrenergic receptor, wherein the peptide inhibits agonist promoted stimulation of adenylyl cyclase activity. These results are completely unexpected in view of the fact that receptor aggregation is not considered to relate to activity of GPCRs.

The present invention also provides for novel peptides modelled on the transmembrane region of GPCRs that can selectively modulate the function of such receptors by affecting the ratio of receptor monomer to multimeric forms (homo-meric or hetero-meric) that can be greater than 20 amino acid residues but less than 50 amino acid residues.

The present invention also provides one skilled in the art with the ability to model a transmembrane domain of GPCRs which form multimers for activity in order to generate novel peptides or peptidomimetic compounds that selectively affect receptor oligomerization (eg. dimerization), thereby selectively affecting receptor function.

The peptides and peptidomimetic compounds of this invention may be utilized in compositions and methods for specifically controlling certain GPCR activities. The invention also involves a process for affecting GPCR activity in mammals which comprises: administering to a subject an effective amount of the novel compound to affect GPCR activity.

A further embodiment involves a pharmaceutical preparation for treating disease and psychoses which comprises administering a pharmaceutically effective amount of the novel peptide or peptidomimetic compound, with a suitable pharmaceutical carrier, sufficient to affect GPCR.

Another aspect of this invention involves generating peptides and peptidomimetic compounds that are useful for *in vitro* and *in vivo* studies of GPCRs.

Due to the fact that the compounds of the present invention may be prepared by chemical synthesis techniques, commercially feasible amounts may be produced inexpensively. Moreover, because the compounds of the present invention are relatively small and may be peptidergic in nature, they are less likely to stimulate an undesirable immune response in patients treated with them.

BRIEF DESCRIPTION OF THE DRAWINGS

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following preferred embodiments of the invention taken in connection with the accompanying drawings in which:

Figure 1 shows immuloblotting of human β_2AR expressed in Sf9 cells. Crude membrane preparations (lane 1), digitonin-solubilized membrane proteins (lane 2) and affinity purified receptors (lane 3) derived from Sf9 cells expressing either c-*myc* tagged (lane 3) or HA-tagged (lanes 1 and 2) β_2AR were immunoblotted following SDS-PAGE using the appropriate antibody (9E10 and 12CA5,

respectively). The blots reveal immunoreactive bands corresponding to the expected monomeric form (43-50kDa) as well as a higher molecular weight species (85-95 kDa). The right panel illustrates immunoblots of crude membrane preparations derived from Sf9 cells expressing HA-tagged β_2AR treated (lane 5) or not (lane 4) with the membrane-permeant photoactivatible crosslinker BASED. Position of receptor bands are denoted by arrows and molecular weight markets are as shown.

Figure 2 shows effects of various peptides and β_2AR ligands on receptor dimerization. Co-immunoprecipitation of β_2ARs bearing two different immunological tags. Lanes 1 and 2: c-*myc* (lane 1) or anti-HA (lane 2) mAbs. The two immunoprecipitates were then immunoblotted with the anti-HA mAb. The occurrence of dimerization between the HA- and c-*myc*-tagged receptors is revealed by the fact that the HA-tagged β_2AR is co-immunoprecipitated with the c-*myc* tagged receptor by the anti-c-*myc* mAb (lane 1). Lanes 3 and 4: c-*myc* tagged β_2AR was expressed in Sf9 cells and immunoprecipitated with anti-c-*myc* mAb. The immunoprecipitates were then immunoblotted with either anti-HA (lane 3) or anti-c-*myc* or anti-c-*myc* (lane 4) mAbs. Lanes 5 and 6: HA-tagged β_2AR was expressed in Sf9 cells, immunoprecipitated with anti-HA mAb and then immunoblotted with either anti-c-*myc* (lane 5) or anti-HA (lane 6) mAbs. These controls demonstrate the specificity of each antibody towards their respective targets. Lane 7 and 8: HA-tagged β_2AR and c-*myc* tagged M2 muscarinic receptors were co-expressed in Sf9 cells, immunoprecipitated with either anti-HA (lane 7) or anti-c-*myc* (lane 8) mAbs. Immunoblotting with the anti-c-*myc* mAb did not reveal the presence of a β_2AR/M2 muscarinic receptor heterodimer (lane 8). Results shown are representative of three separate experiments.

Figure 3 demonstrates Immunoblotting of V2-vasopressin receptors (V2-R) expressed in COS-7 cells. Crude membrane preparations from COS-7 cells transiently transfected with c-*myc* tagged V2-R (lane 1) or c-*myc* tagged V2-R truncation mutant o11 (lane 2) were immunoblotted with the anti-c-*myc* mAb. The molecular weight markets are as shown. Square brackets highlight the dimeric species of both wildtype and O-11 V2 vasopressin receptors while asterisks denote the monomeric species. Data are representative of three independent experiments.

Figure 4 shows effects of various peptides on receptor dimerization. A, Time course of the effect of the TM VI peptide on β_2AR dimerization. Membranes derived from Sf9 cells expressing β_2AR were treated at room temperature with TM VI peptide [residues 276-296: NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH] at a concentration of 0.15 µg/µL for 0 (lane 1), 15 (lane 2), 20 (lane 3) or 30 minutes (lane 4). Membranes were then subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with the anti-c-*myc* antibody. A representative immunoblot is shown. B, Densitometric analyses of three experiments similar to that shown in the Figure 4a demonstrating the effects of treatment for 30 minutes with either vehicle (CON, lane 1), TM VI peptide (TM VI, lane 2) TM VI-Ala [NH_2-AIIMATFTACWLPFFIVNIVH-COOH] (TM VI-Ala, lane 3), or D2 dopamine receptor TM VII peptide [residues 407-426 NH_2-YIIPNVASNVYGLWTFASYL-COOH) (D2 TM VII, lane 4). All peptides were used at a concentration of 0.15 µg/µL. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. Data shown are mean +/- SEM (n=3).

Figure 5 demonstrates, in A, effects of increasing concentrations of TM VI peptide on the amount of β_2AR dimer. Increasing concentrations (0-6.3 mM) of the peptide were added to purified c-*myc* tagged β_2AR and the amount of dimer assessed by immunoblotting using the anti c-*myc* mAb (lanes 1 - 8). In lanes 9 and 10 purified β_2AR was treated (lane 10) or not (lane 9) with the D2 TM VII peptide. The data shown are representative of three distinct experiments. Other control peptides used to determine the selectivity of the effect observed with the TM VI peptide included one derived from the C-terminal tail of the β_2AR [residues 347-358 NH_2-LKAYGNGYSSNG-COOH] or an additional control peptide unrelated to the β_2AR but of similar size as the TM VI peptide [NH_2-SIQHLSTGHDHDDVDVGEQQ-COOH] were also found to be without effect on the amount of dimer (data not shown). B, Densitometric analyses of three experiments similar to that shown in B. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. *Inset* shows superimposed densitometric scans of immunoblotted receptors which were previously treated with increasing concentrations of the TM VI peptide. The monomer is denoted by M while the dimeric species is marked by D. The concentration of peptide added for the curves shown was: none (.....), 0.07 mM (---...----), 0.05 mM (--- ---), and 1.25 mM (------).

Figure 6 demonstrates effects of TM VI peptide on β_2AR stimulated adenylyl cyclase activity in Sf9 cells. A, Membrane preparations derived from β_2AR expressing Sf9 cells were either not treated (open circles), or treated with TM VI peptide (closed squares), control peptide TM VI Ala (closed circles), or second control peptide from TM VII of the D2 dopamine receptor (open triangles). Isoproterenol stimulated adenylyl cyclase activity was then assessed for these membranes. Data are expressed relative to the maximal stimulation obtained with the untreated membranes and represent mean +/- SEM for 8 independent experiments. Peptides were used at a concentration of 0.15 μg/μl. B, Effects of TM VI peptide (hatched bars) or vehicle alone (open bars) on basal (n = 13), maximal isoproterenol-stimulated (ISO, n = 13), forskolin-mediated (FSK, n = 13) and NaF-stimulated (n = 6) adenylyl cyclase activity was investigated. Data are expressed as pmol cAMP produced per mg membrane protein per minute +/- SEM. Statistical significance of the difference are indicated by an asterisk and represent a $p<0.05$ as assessed by a non-paired student's t-test. None of the control peptides discussed in figure 2 had effects on adenylyl cyclase stimulation in β_2AR expressing cells nor did any of the peptides have effects on adenylyl cyclase stimulation in Sf9 cells which were infected with the wildtype baculovirus (data not shown). C, Effects of increasing concentrations of peptide on isoproterenol and dopamine stimulated adenylyl cyclase activity were also investigated. Membranes were prepared from Sf9 cells expressing either the human β_2AR (open circles) or the human D1 dopamine receptor (closed circles). Adenylyl cyclase activity was measured using maximally stimulating concentrations of either isoproterenol (10^{-4} M) or dopamine (10^{-4} M) in the presence of peptide concentrations ranging from 10^{-8} to 10^{-4} M. Data were analyzed by non-linear least squares regression using SigmaPlot (Jandel Scientific). The data are expressed as the mean +/- SEM (n = 3).

Figure 7 shows effects of β_2AR ligands on receptor dimerization. A, Time course of the effect of 1 μM isoproterenol on β_2AR dimerization. Membranes derived from Sf9 cells expressing the c-*myc* β_2AR were treated at room temperature with 1 μM isoproterenol for 0 (lane 1), 15 (lane 2), 20 (lane 3) or 30 minutes (lane 4). Membranes were then subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with the anti-c-*myc* antibody. A representative immunoblot is

shown. B, Densitometric analyses of three experiments where membranes from Sf9 cells expressing the β_2AR were treated for 30 minutes at room temperature with either vehicle (CON), 1 μM isoproterenol (ISO), 10 μM timolol (TIM), TM VI peptide at a concentration of 0.15 μg/μL (TM VI), or isoproterenol followed by 30 minutes with Tm VI peptide (ISO/PEP). The TM VI data (lane 4) is reproduced from Figure 4b for comparison. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. Data shown are mean +/- SEM (n=3).

Figure 8 depicts effects of TM VI peptide on β_2AR expressed in mammalian cells. A, Effect of 0.15 ug/ul TM VI peptide (hatched bars) or vehicle (open bars) on basal (n=2), maximal isoproterenol-stimulated (ISO, n=2) forskolin-mediated (FSK, n=2) and NaF-stimulated (NaF, n=2) adenylyl cyclase activity in CHW cells expressing 5 pmol β_2AR/mg protein. Data are expressed as pmol cAMP produced per mg membrane protein per minute ± SEM. Statistical significance of the difference are indicated b an asterisk and represent a $p<0.05$ as assessed by a non-paired student's t-test. Membranes were treated with either vhicle (lane 1) or the TM VI peptide at a concentration of 0.15 ug/ul (lane 2) for 30 minutes at room temperature. Membranes from untransfected CHW cells had no detectable receptors (data not shown). **B**, Effects of TM VI peptide on β_2AR stimulated adenylyl cyclase activity in mouse Ltk$^-$ cells. Membranes were prepared from Ltk$^-$ cells stably expressing 200 fmol of human β_2AR/mg membrane protein. Isoproterenol-stimulated adenylyl cyclase activity was then assessed in membranes treated with vehicle (open circles), TM VI peptide (closed squares), control peptide TM VI Ala (closed circles), or the D2 TM VII control peptide (open triangles). Data are expressed relative to the maximal stimulation obtained with vehicle treated membranes and represent mean ± SEM for 3 independent experiments. Peptides were used at a concentration of 0.15 ug/ul.

Figure 9 demonstrates sequence data collated from numerous published articles oriented to compare the peptide sequences of the putative TM VI regions of twenty-seven GPCRs. While hydropathy analysis may yield results of uncertain reliability when identifying the TM VI and TM VII domains of particular groups of GPCR, sequence analysis can identify the "GGL motif," corresponding to TM

VI, with greater certainty.

DETAILED DESCRIPTION OF THE INVENTION

The following common abbreviations are used throughout the specification and in the claims:
The abbreviation, IP is inositol phosphate.
The abbreviation BASED is bis [β-(4 azidosalicylamindo) ethyl] disulphide.
The abbreviation, 5-HT is 5-hydroxytryptamine.
The abbreviation, DOI is 2,5-dimethoxy-4-iodoamphetamine hydrobromide.
The abbreviation, PBS is phosphate buffered saline.
The abbreviation, β_2AR is β_2-adrenergic receptor.
The abbreviation, GPCR is G protein-coupled receptor.
The abbreviation, GpA is glycophorin A.
The abbreviation, HA is influenza hemagglutinin.
The abbreviation TM VI is transmembrane domain 6.
The abbreviation, NDI is nephrogenic diabetes insipidus.

Orphan receptors are receptors for which the natural ligands and/or biological function are uncertain or unknown.

The term "any amino acid" as used herein includes the L-isomers and D-isomers of the naturally occurring amino acids, as well as other "non-protein" α-amino acids commonly utilized by those in the peptide chemistry arts when preparing synthetic analogues of naturally occurring peptides. The naturally occurring amino acids are glycine, alanine, valine, leucine, isoleucine, serine, methionine, threonine, phenylalanine, tyrosine, tryptophan, cysteine, proline, histidine, aspartic acid, asparagine, glutamic acid, glutamine, Y-carboxyglutamic acid, arginine, ornithine and lysine. Examples of "non-protein" -amino acids include norleucine, norvaline, alloisoleucine, homoarginine, thioproline, dehydroproline, hydroxyproline (Hyp), isonipecotic acid (Inp), homoserine, cyclohexylglycine (Chg), -amino-n-butyric acid (Aba), cyclohexylalanine (Cha), aminophenylgutyric acid (Pba), phenylalanines

substituted at the ortho, meta, or para position of the phenyl moiety with one or two of the following: a (C_1-C_4) alkyl, a (C_1-C_4) alkoxy, halogen or nitro groups or substitute with a methylenedioxy group, β-2- and 3-thienylalanine, β-2- and 3-furanylalanine, β-2-, and 3- and 4-pyridylalanine, β-(benzothienyl-2- and 3-yl)alanine, β-(1- and 2-napthyl)alanine, O-alkylated derivatives of serine, threonine or tyrosine, S-alkylated cysteine, S-alkylated homocysteine, O-sulfate, O-phosphate and O-carboxylate esters of tyrosine, 3-sulfo-tyrosine, 3-carboxy-tyrosine, 3-phospho-tyrosine, 4-methane sulfonic acid ester of tyrosine, 4-methane phosphonic acid ester of tyrosine, 3,5-diiodotyrosine, 3-nitrotyrosine, ϵ-alkyl lysine, delta-alkyl ornithine, and the ϵ-isomers of any of the above amino acids. Unless specifically indicated, all amino acids referred to in this application are in the L-form. The term "positively charged amino acid" as used in this application includes any naturally occurring or non-naturally occurring amino acid having a positively charged side chain. Examples of positively charged amino acids are arginine, lysine, histidine, homoarginine, ornithine and delta-alkyl ornithine.

The term "amino acid containing an aryl side chain" as used herein means any amino acid having an aromatic group. Tyrosine, phenylalanine, tryptophan, O-sulfate esters of tyrosine and 5-nitrotyrosine exemplify such amino acids.

The term "polar amino acid" means any amino acid having an uncharged side chain which is relatively soluble in water. Examples include glutamine, asparagine, glycine, serine, hydroxyproline and homoserine.

The term "hydrophobic amino acid" means any amino acid having an uncharged side chain which is relatively insoluble in water. This group includes leucine, valine, tryptophan, norleucine, norvaline, alloisoleucine, thioproline, dehydroproline, cyclohexylalanine and cyclohexylglycine.

The term "patient" as used in this application refers to any mammal, especially humans.

The term "backbone chain" as used herein, refers to the portion of a chemical structure that defines the smallest number of consecutive bonds that can be traced from one end of that chemical structure

to the other. The atomic components that make up a backbone chain may comprise any atoms that are capable of forming bonds with at least two other atoms.

The term "peptide-lead" as used herein, refers to the undervitized peptide that is modelled on one of the transmembrane domains in the first step of the design of the compounds of this invention. Examples of peptide-leads are listed in Example III.

The term "parent GPCR" as used herein, refers to the GPCR from which the peptide-lead is derived. Molecules of this invention are designed using the peptide-lead as the model for GPCR-peptides that are selectively inhibitory towards the parent GPCR or closely related receptors.

The term "GPCR-peptide" as used herein, refers to peptides, modified or unmodified, which have been designed and synthesized according to the methods of this invention, that can be used to selectively prevent or disrupt functional aggregation of GPCR's which form multimers (eg. dimers) for activity.

The term "oligomeric GPCR" refers to G protein-coupled receptors that form oligomers (eg. dimers) and that such interactions have functional consequences. Oligomers can be homo-multimeric or hetero-multimeric.

The terms "selective inhibition" and "selectively inhibits" as used herein, refers to the ability of the peptide to inhibit the actions of a species of GPCR and/or closely related receptors without affecting the activity of other non-related species of receptors to any significant extent. Significant extent means there must be at least a 10-fold magnitude difference in inhibitory activities between the parent receptor and other types of receptors.

The present invention relates to novel peptides and/or peptidomimetic compounds that are modeled on the transmembrane region of GPCRs. The peptides and peptidomimetic molecules of this invention selectively bind to the GPCR from which they were designed. This property allows the

molecules of this invention to affect those activities of the GPCR that are mediated through this receptor.

Applicant has discovered that peptides corresponding to residues 276-296, residing in the 6th transmembrane region of the β_2-adrenergic receptor, selectively inhibit agonist promoted stimulation of adenylyl cyclase activity. Applicant believes that this peptide forms structural complimentarity with the receptor from which it was derived, preventing the receptor from interacting with the molecules necessary to effect its activities. Peptides and other molecules which comprise such features will bind to the receptor from which the peptide was derived and affect its activation *in vivo* and *in vitro*. These peptides and other molecules, as well as compositions and methods which employ them, make up the present invention.

According to one embodiment, the GPCR-peptides of the present invention are characterized by complimentarity to one of the transmembrane domains of the parent receptor. Preferably, that region is modelled on one of the transmembrane domains. The GPCR-peptides of this invention are further characterized by: (1) the ability to affect (positively or negatively) the activity of the parent GPCR and possibly closely related receptors, and (2) the inability to significantly affect other types of receptors (i.e. other receptors can be inhibited if there is a 10-fold magnitude difference in activities)

Determination of Candidate Transmembrane Sequences (TMS)

Each transmembrane sequence provides a potential model for a peptide-lead which will be used to design peptides or peptidiomimetic compounds that could function to disrupt or prevent functional aggregation of the same GPCR (parent-GPCR) and possibly closely related receptors. Thus, the first step is to identify the transmembrane domain of the receptor of interest. There are a number of techniques well known in the art available for determining transmembrane regions of the GPCR. These include hydropathy plots to identify the hydrophobic segments. The secondary structure can also be analyzed to identify alpha-helix structures. This information can be used in combination with

the general view that GPCRs are organized in a manner similar to bacteriorhodopsin, which has been shown to possess seven transmembrane alpha-helical hydrophobic regions. Moreover, there are many software packages that will automatically generate a multiple sequence alignment for almost any set of amino acid sequences of GPCRs.

Since GPCRs of the family A/rhodopsin related subfamily share a number of features common to all members of its class. One of these features is the presence of recurring patterns in their amino acid sequence. Each transmembrane domain (TM) can be characterized by a recurring pattern that is unique for that TM. The alignment of the TMs is therefore based on recurring patterns rather than on homology alone. For example, in a preferred embodiment such as presented in Example III, the patterns used were:

TM1: GXXXN or GN
TM2: LXXXDXXXXXXXP or LXXXDXXXXXXXXP
TM3: SXXXLXXIXXDR or SXXXLXXI XXHR
TM4: WXXXXXXXXP or WXXXXXXXXXP
TM5: FXXPXXXXXXXY
TM6: FXXCXXP
TM7: LXXXXXXXDPXXY or LXXXXXXXNPXXY

To identify a position within an alignment, rather than a position within a specific amino acid sequence, a "sequence identifier" is assigned to each position in the alignment. To obtain the "sequence identifier" for a particular residue (listed at the bottom of each column in the alignments), the "extended notation" convention is applied. This reads as follows:

3 |This is the TM identifier.
2 |The last two digits indicate the residue number relative to
9 |the most conserved residue, which is number 50 by default.

This is the equivalent of, e.g., R110(3.29) in the hP2U sequence, S105(3.29) in the hAT1A sequence, or T114(3.29) in the hbeta3 sequence. A more comprehensive description of this convention can be found in: Ballesteros, J.; Weinstein, H., Meth. Neurosci., 1995, 25: 366-428. van Rhee, A.M.; Jacobson, K.A., Drug Devel. Res., 1996, 37: 1-38 (On-line as of February 28, 1995 and the last update was performed on September 27, 1996).

Methods for studying hydropathy using computer-assisted structural assessment techniques (Kyte, J., and Doolittle, R.F., *J. Mol. Biol.*, 157:105-132, 1982), flexibility (Karplus, P.A., and Schultz, G.E., *Naturwissenschaften*, 72:212-213, 1985), and secondary structure (Chou, P.Y., and Fasman,G.D., *Annu. Rev. Biochem.*, 47:251-276, 1976) of the trans-membrane domains are well known in the art (for eg., see: Krystek et al., *Endocrinology* (Baltimore) 117:1125-1131, 1985a; *Endocrinology* (Baltimore) 117:1110-1124, 1985b). Surface profiles (eg. the Kyte and Doolittle scale) can be used to identify regions of the GPCR which should be accessible for protein-protein interactions, with the caveat that such analysis does not take into account the carbohydrate chains located on proteins, and disulfide bonds are not adequately analyzed by these methods. Correlation of flexibility plots with homology plots and surface profiles may be helpful in identifying specific regions of protein structure. The most flexible regions of a protein are reportedly associated with protein binding sites (Richardson, J.S., *Adv. Protein Chem.* 34, 167-339, 1981; Van Regenmortel, M.H.V., *Trends Biochem. Sci.*, 237-240 , 1987). Flexibility plots ostensibly identify the most flexible regions of a polypeptide but do not take into account disulfide bonding (Spinella, M.J. et al., *Pept. Res.* 2, 286-291, 1989) Since disulfides are known to stabilize protein structures (Richardson, J.S., *Adv. Protein Chem.* 34, 167-339, 1981), and the flexibility of a molecule that is highly disulfide bonded is probably overestimated by this technique, this limitation should be kept in mind.

In some situations, it may not be desirable to use the hydropathy methods for determining transmembrane (TM) sequences. For example, in the sixth and seventh transmembrane (TM VI and TM VII, respectively) domain regions, the hydrophobic sequence patterns are not as clear. Thus, other methods such as comparisons of a unique amino-acid motif found within one successful peptide-lead may be useful.

By assessing the similarities and distinctions of a unique amino acid motif of the GPCR of interest (such as the GGL motif found within the TM VI sequence of the β_2AR) one skilled in the art could predict with which groups of receptors within the GPCR family a polypeptide of a particular amino acid sequence might interact more or less strongly, and with which ones that same polypeptide would be unlikely to interact. Thus one skilled in the art might also be able to select a sequence structure for a peptide or peptidomimetic compound in order to select the breadth or narrowness of interactions amongst related GPCRs, as might be desired.

For example, while hydropathy analysis may yield results of uncertain reliability when identifying the TM VI and TM VII domains of particular groups of GPCR, sequence analysis identifies a "GGL motif", corresponding to TM VI, with greater certainty. Data presented in Figure 9 demonstrates that the reproducibility of the GGL motif between GPCRs is grouped into subgroups of receptors with similar functions. The GGL motif is located in a transmembrane region of the GPCR that is not known to be involved in any of the domains recognized as participating in protein-protein interactions within the signal transduction complex. A possible relationship exists between the GGL sequence and GPCR specificity which could be compatible with a self-recognition role for this domain. The relationship of the closeness of function/family grouping of GPCR to the similarity of the TM VI / GGL motif's sequences suggests that the breadth of the potential or probable interactions between the peptide with the β_2-AR TM VI sequence and the equivalent sequence (GGL motif) of any other GPCR, might be predictable. This in turn provides a possible method of predicting the selectivity (or otherwise) of the action of any particular TM VI peptide.

Design and Synthesis of Peptides Modeled on TMS

In designing a GPCR-peptide according to this invention, two important considerations must be taken into account. First, the molecule must be able to physically associate with the parent-GPCR. The present theory of peptide binding suggests that the initial step in binding requires, at a minimum, an ionic interaction between the receptor and the peptide. It is also probable that other molecular

interactions, such as hydrogen bonding and hydrophobic interactions, are important for this association. Therefore, the identification and maintenance of these interactions are critical in designing a potent GPCR antagonist.

The second consideration in designing the GPCR-peptides of this invention is secondary and tertiary structure. While certain portions of the GPCR-peptide will not directly participate in molecular interactions with the receptor, they may play a role in the overall conformation of the GPCR-peptide. This, in turn, can have a dramatic effect on potency. If the GPCR-peptide cannot assume the proper conformation, the molecular interactions required for association with the receptor cannot be achieved, even if the components capable of forming such interactions are present in the molecule. Accordingly, GPCR-peptides of this invention must be designed so that they assume a conformation which allows them to associate with the receptor. Conformational requirements may be in the nature of overall three-dimensional structure and orientation of the GPCR-peptide, or merely the spacing between two sites on the GPCR-peptide which directly interact with the receptor.

To test which of the amino acids in the 15-20 amino acid peptide-lead are responsible for crucial molecular interactions with the parent GPCR, a simple alanine scanning procedure is carried out. In this procedure, a series of peptides, each having a single alanine substitution at a different residue, is synthesized. The peptides are then assayed to determine if they selectively inhibit GPCR activity.

Those alanine-substituted peptides which retain an ability to prevent or disrupt functional aggregation of GPCRs which form multimers for activity, indicate portions of the peptide-lead that do not directly interact with the receptor and which do not have side chains which play a critical role in the folding of the GPCR-peptide. Such peptides are preferred peptide-leads and GPCR-peptides of the present invention. Conversely, those peptides which lack or have greatly reduced disruptive activity point out areas of the peptide-lead and GPCR-peptide that are important for activity. These latter peptides suggest the nature of an important interior intramolecular interaction based upon the amino acid substituted for. For example, an arginine-to-alanine substitution which resulted in reduced activity suggests the location of an important positive charge - either an ionic interaction with the

receptor or an intramolecular ionic interaction within the peptide - which is required to maintain optimal conformation. A serine-to-alanine substitution which had a negative effect on activity indicates the location of an important hydrogen bond. Again, the hydrogen bond may be between the peptide-lead and the GPCR, or it may be an intramolecular hydrogen bond that plays an important role in the conformation of the peptide-lead.

Those of skill in the art will realize that distinguishing between whether a structural feature is important for an inter- or intramolecular interaction can only be achieved by examining an X-ray crystal structure of the peptide-receptor complex. However, that distinction is of little import in designing the peptide-leads and GPCR-peptides of this invention. Once the nature of the interaction is determined. *i.e.*, electrostatic, hydrophobic, ionic, the choice of potential substitutes at that position becomes clear.

To further ascertain those sites that are important for proper folding and orientation of the peptide-leads and GPCR-peptides of this invention, a single position deletion analysis is performed. In this procedure, a series of peptides containing single deletions at positions which do not affect inhibitor activity are synthesized and assayed for activity. The peptides from this series that retain significant activity indicate areas of the peptide-lead and GPCR-peptide that are not essential for proper conformation. Such peptides are also included within the scope of this invention.

Deletion peptides from this series which have significantly lower attenuating activity indicate the location of components which provide critical spacing in the peptide-lead or GPCR-peptide. This may be verified by replacing the deleted amino acid with a different, yet analogous structure. For example, substitution of any conformationally important amino acid with a three carbon alkyl chain without a significant loss of activity confirms that spacing is critical at that part of the molecule.

Additional information about important structural and conformational features necessary for designing a potent GPCR-peptide of this invention may be obtained through 3-dimensional X-ray crystallographic procedures coupled with computer modelling. Specifically, one of ordinary skill in

the art may analyze a GPCR/peptide-lead using such a method. Alternatively, one of average skill in the art could employ multiple alanine substitutions or multiple deletions to identify important intramolecular interactions in the antagonist itself. It will also be apparent that each new GPCR-peptide designed and tested will, itself, provide additional information about structural features important for inhibition of GPCR activity.

Once the critical residues in the peptide-lead have been located and characterized, other GPCR-peptides of this invention may be designed and synthesized. This is achieved by substituting the identified key residues of the peptide-lead with other components having similar features. These substitutions will initially be conservative, *i.e.*, the replacement component will have approximately the same size, shape, hydrophobicity and charge as the key residue. Those of ordinary skill in the art are well aware of appropriate replacements for a given amino acid [Dayhoff et al., in *Atlas of Protein Sequence and Structure No. 5*, 1978 and Argos et al., *EMBO J.*, 8, pp. 779-85 (1989)]. Typical conservative substitutions for an amino acid are other amino acids with similar charges, for example, aspartic acid for glutamic acid, arginine for lysine, asparagine for glutamine, hydroxyproline for proline and *vice versa*. Substitutions with non-natural amino acids may also be performed to reduce the peptidic nature of the peptide-lead. Some examples are cyclohexylalanine for tyrosine, sarcosine for glycine, statine for threonine and homoarginine for arginine. These modifications may increase the biological stability of the antagonist, in addition to increasing its potency.

After the molecule containing the substitute component is shown to be a compound effective for selectively preventing or disrupting the aggregation of GPCRs, less conservative replacements may be made at the same position. These substitutions typically involve the introduction of non-amino acid components which contain the important feature imparted by the amino acid at that position. Such substitutes are well-known in the art. For example, the sequence Leu-Val-Arg (corresponding to amino acids 65-67 of thrombin) can be replaced by p-guanidinobenzoic acid. This substitution maintains the hydrophobicity of Leu-Val, as well as the guanidinium functionality of Arg.

It will be apparent that there is greater freedom in selecting the substitute for a non-essential amino

acid in the peptide-lead sequence. Moreover, a non-essential amino acid may simply be eliminated. Almost any substitute that does not impart a change in conformation may be employed for a nonessential amino acid. These include, but are not limited to, straight chain alkyl and acyl groups. Also, because of the importance of the net positive charge of the antagonist, anionic substitutes should be avoided. Components which are known in the art to alter conformation should also be avoided. One such component is proline, an amino acid which causes a turn structure in a molecule. Others are well-known in the art [G.D. Rose et al., "Turns in Peptides and Proteins", Adv. Prot. Chem., 37, pp. 1-110 (1985)].

In addition to those peptides resulting from the substitutions and deletions described above, novel GPCR-peptides according to this invention may be designed by insertions at various sites along the peptide-lead. To determine areas of the peptide-lead where a component may potentially be inserted, a series of peptides having a single alanine insertion at various sites is synthesized. Those peptides from this series which retain activity for selectively preventing or disrupting functional aggregation of GPCRs which form multimers for activity indicate potential insertion sites.

In choosing a component to be inserted, one should be guided by the same considerations set forth above in selecting a substitute component. Specifically, one must keep in mind how the insertions may potentially affect the molecular interactions between the GPCR-peptide and the GPCR and how they affect conformation of the GPCR-peptide. For example, the insertion of an anionic component adjacent to a critical cationic amino acid in the peptide-lead could interfere with an important ionic interaction and should therefore be avoided. Similarly, the insertion of a component which is known to cause structural perturbations, e.g., a proline, should also be avoided.

Using any or all of the above deletion, substitution and insertion techniques allows those of ordinary skill in the art to design GPCR-peptides according to this invention. Moreover, the potential effect of any of these changes may be theoretically observed prior to synthesizing the GPCR-peptide through the use of computer modelling techniques known in the art. Such modelling allows one to observe the predicted structure of a GPCR complexed with the potential GPCR-peptide. If that

theoretical structure suggests insufficient interaction between the receptor and the potential GPCR-peptide, one need not spend time and resources in synthesizing and testing the molecule. On the other hand, if computer modelling indicates a strong interaction, the molecule may then be synthesized and assayed for activity. In this manner, inoperative molecules may be eliminated before they are synthesized.

Finally, cyclic derivatives of any peptide designed by the above techniques are also part of the present invention. Cyclization may allow the peptide to assume a more favorable conformation for association with the GPCR. Cyclization may be achieved by methods well-known to those in the art. One method is the formation of a disulfide bond between two non-adjacent cysteine residues (D- or L-conformation) or any two appropriately spaced components having free sulfhydryl groups. It will be understood that disulfide bonds, as well as other intramolecular covalent bonds, may be formed between a variety of components within the GPCR-peptide. The components which form such bonds may be side chains of amino acids, non-amino acid components or a combination of the two.

The most preferred peptides of the present invention are modelled after the peptide-lead which comprises the formula: SEQ ID NO: 1: NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH. In order to ensure the entire predicted region be contained in a single peptide, it may be wise to extend both the amino terminus and the carboxyl terminus.

In addition to the above sequence, a variety of other peptide-leads modelled on the transmembrane domain sequences of GPCRs can be used as set out in Examle III, below.

The synthesis of the peptides of this invention including derivation, activation, and coupling of protected amino acid residues, and their purification, and the analytical methods for determining identity and purity are included in the general body of knowledge of peptide chemistry, as described in Houben Weyl *Methoden der Organischen Chemie*, (1974), Vol. 16, parts I & II for solution-phase synthesis, and in *Solid Phase Peptide Synthesis*, (1984), by Stewart and Young for synthesis by the solid-phase method of Merrifield.

Any chemist skilled in the art of peptide synthesis can synthesize GPCR peptides of this invention by standard solution methods. These include enzymatic cleavage of GPCR, recombinant DNA techniques, solid-phase peptide synthesis, solution-phase peptide synthesis, organic chemical synthesis techniques, or a combination of these techniques. The choice of synthesis technique will, of course, depend upon the composition of the particular peptide. In a preferred embodiment of this invention, the GPCR peptide is entirely peptidic and is synthesized by solid-phase peptide synthesis techniques, solution-phase peptide synthesis techniques or a combination thereof which constitute the most cost-efficient procedures for producing commercial quantities of these peptides.

When "non-protein" amino acids are contained in the GPCR peptide, they may be either added directly to the growing chain during peptide synthesis or prepared by chemical modification of the complete synthesized peptide, depending on the nature of the desired "non-protein" amino acid. Those of skill in the chemical synthesis art are well aware of which "non-protein" amino acids may be added directly and which must be synthesized by chemically modifying the complete peptide chain following peptide synthesis.

The synthesis of those GPCR peptides of this invention which contain both non-amino acid and peptidic portions is preferably achieved by a mixed heterologous/solid phase technique. This technique involves the solid-phase synthesis of all or most of the peptide portion of the molecule. This is followed by the addition of the non-amino acid components which are synthesized by solution phase techniques and then coupled to the peptidic portion via solid-phase or solution-phase methods. Any remaining peptidic portions may then be added via solid-phase or solution-phase methods.

In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various n-protecting groups, e.g., the carbobenzyloxy group or the t-butyloxycarbonyl group (BOC), various coupling reagents, e.g., dicyclohexylcarbodiimide or carbonyldimidazole, various active esters, e.g., esters of N-hydroxypthalimide or N-hydroxy-

succinimide, and the various cleavage reagents, e.g., trifluoracetic acid (TFA), HCl in dioxane, boron tris-(trifluoracetate) and cyanogen bromide, and reaction in solution with isolation and purification of intermediates is well-known classical peptide methodology.

A preferred peptide synthesis method follows conventional Merrifield solid-phase procedures. See Merrifield, *J. Amer. Chem. Soc.* 85, 2149-54 (1963) and *Science* 150, 178-85 (1965). This procedure, though using many of the same chemical reactions and blocking groups of classical peptide synthesis, provides a growing peptide chain anchored by its carboxy terminus to a solid support, usually cross-linked polystyrene, styrenedivinyl-benzene copolymer or, preferably, p-methylbenzhydrylamine polymer for synthesizing peptide amides. This method conveniently simplifies the number of procedural manipulations since removal of the excess reagents at each step is effected simply by washing the polymer.

Further background information on the established solid phase synthesis procedure can be had by reference to the treatise by Stewart and Young, *"Solid Phase Peptide Synthesis,"* W.H. Freeman 7 Co., San Francisco, 1969, and the review chapter by Merrifield in *Advances in Enzymology* 32, pp. 221-296, F.F. Nold, Ed., Interscience Publishers, New York, 1969; and Erickson and Merrifield, *The Proteins*, Vol 2, p. 255 et seq. (ed. Neurath and Hill), Academic Press, New York, 1976.

Design of Peptidomimetic Compounds

It is well known in the drug design art to look for a substitute compound that mimics the conformation and desirable features of a particular peptide, e.g., an oligopeptide, once such peptide has been found, but that avoids the undesirable features of a peptide compound, e.g., flexibility (loss of conformation) and bond breakdown. Such a compound that mimics a peptide is known as a "peptidomimetic". There are a number of methods for designing peptidomimetic compounds that are known in the art.

In each case, the starting point for designing a peptidomimetic compound is the sequence

and/or conformation of a particular oligopeptide or peptide of interest. For example, see, Spatola, A.F. *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins* (Weistein, B, Ed.), Vol. 7, pp. 267-357, Marcel Dekker, New York (1983), which describes the use of methylenethio bioisostere [CH_2S] as an amide replacement in enkephalin analogues; and Szelke et al., *In Peptides: Structure and Function, Proceedings of the Eighth American Peptide Symposium,* (Hruby and Rich, Eds.); pp. 579-582, Pierce Chemical Co., Rockford, Ill. (1983), which describes renin inhibitors having both the methyleneamino [CH_2NH] and hydroxyethylene [$CHOHCH_2$] bioisosteres at the Leu-Val amide bond in the 6-18 octapeptide derived from angiotensinogen.

It is also known in the art to use computer simulation in an attempt to predict a stable conformation of a peptide. That is, because a peptide is a sequence of amino acid residues, each containing known atoms bonded together in known molecules having known bonding lengths, with known electrostatic properties associated with each atom, it is possible to simulate a peptide structure on the computer. An example of such a computer based method of rational drug design that identifies bioactive peptidomimetics that can be effectively used as drugs is U.S. Patent No. 5,331,573. This method predicts the most probable secondary and/or tertiary structures of a polypeptide without any presumptions as to the conformation of the underlying primary or secondary structure. The method involves computer simulation of the peptide in a manner that simulates a real-size primary structure in an aqueous environment, shrinking the size of the polypeptide isobarically and isothermally, and expanding the simulated polypeptide to its real size in selected time periods. A useful set of tools, terms Balaji plots, energy conformational maps and probability maps, assist in identifying those portions of the predicted peptide structure that are most flexible or most rigid. The overall method of this technique involves the following steps:

(a) simulating the most probable conformations of a given polypeptide;

(b) selecting the most probable conformation of the peptides thus simulated;

(c) designing and synthesizing a chemically modified analog of the selected peptide;

(d) evaluating the bioactivity of the synthesized chemically modified analog of the selected peptide; and thereafter, optionally

(e) designing a suitable peptidomimetic based on the conformation of the synthesized

chemically modified analog of the selected peptide.

In carrying out this method, if it is noted that the chemically modified analog of the selected peptide is not bioactive, as determined through suitable testing, then an additional step relates to determining whether other chemically modified analogs should be designed for this same selected peptide. If so, then another chemically modified analog for the selected peptide is designed and the bioactivity of this newly designed chemically modified analog is evaluated. If a determination is made that another chemically modified analog for this same peptide should not be designed, then the nest most probable conformation of the simulated peptide is selected and a chemically modified analog is designed and synthesized for such selected peptide and the process is repeated.

Experimental Assessment of Candidate Peptides

The ability of the peptides and peptidomimetic compounds of this invention to selectively affect oligomerization of GPCRs which form multimers for activity can be measured by choosing from the many techniques available in the art. In general, the goal of these types of assays would be to measure the ratio of monomeric receptor to multimeric receptors (dimers, trimers, etc.) The change in ratio of the relative amounts of monomer to multimer will reflect conversion of monomers to multimers or *vice versa*. If the results of these assays are correlated with measurements of the activity of the GPCR (using techniques described below), one skilled in the art will be able to ascertain whether the peptide-lead, GPCR-peptide or peptidomimetic compound will interfere with the functional aggregation of receptor subunits which form multimeric associations for functional activity. Those compounds which promote oligomerization would be predicted to have one activity (eg. agonist or positive efficacy) while those which promote dissociation of oligomers would be predicted to demonstrate opposite activity (eg. inverse agonists or negative efficacy). The magnitude of change in ratio and/or rate of change effected by the compound would provide a measure of the compound's efficacy and/or potency in modulating receptor activity.

<u>Measuring the ratio of monomeric receptor to multimeric receptor</u>

There are many different techniques available for determining the relative amount of monomer to multimer (eg. dimer) formed in the presence and absence of the peptide-lead, GPCR-peptide, or peptidomimetic compound. For example, different assay systems can be designed to measure the ability of compounds to modify the ratio of monomers/multimers. In general, any procedure that permits measurement of the relative amounts of monomer and oligomer in receptor preparations (eg. membranes, solubilized receptor preparations, purified receptors, etc) can be used. Typically, a sample containing the compound to be tested or a control sample lacking the compound would be added to a suspension or solution of receptor preparation. After an incubation period, the receptor preparation would be analyzed to determine the relative amounts of monomeric and oligomeric species such that changes in the ratio produced by the test compound could be used to predict the activity and efficacy of the compound in regulating receptor function.

Immunological methods can be used to measure compound efficacy. As demonstrated by the working example provided herein (see Figures and Examples) differential epitope tagging can be used in combination with differential co-immunoprecipitation to demonstrate the formation or absence of multimeric subunit aggregation. As each type of subunit bears a unique tag, immunological techniques can be used to purify and identify the presence of each subunit in a multimer. If the complex is made up of two or more identical subunits (eg. homodimer or homotrimer), each subunit is treated as if it is unique, such that the subunits bear tags in proportion to the number of units in the multimer. For example, if the complex is a homodimer, one-half of the cDNA will be tagged with tag A and the other-half will be tagged with tag B. The resulting dimers will form between A-A, AB, and BB subunits, but will be observable by their migration in the SDS-PAGE gel, relative to the individual units. These will be visualized by immunoblotting with either or both types of anti-A MAbs or anti-B MAbs.

In a preferred embodiment of this invention, the following steps can be followed:

1) Synthesize sets of recombinant baculoviruses, wherein each set comprises cDNA encoding one subunit of a receptor and one unique immunologic tag, one set for each subunit;

2) co-express the sets of receptor cDNA, each set bearing a unique tag, in Sf9 cells;

3) solubilize membranes and purify receptors;

4) add test compound to the receptor preparation;

5) immunoprecipitate the receptors using anti-tag MAbs, one per unique tag;

6) separate the receptors using SDS PAGE;

7) immunoblot the SDS PAGE gel to observe resultant subunit aggregations;

An immunological method for measuring monomer/oligomer ratio entails separating monomers and oligomers based on size and measurement of relative amounts of each using reporter systems. In this embodiment the following steps would be followed:

1) receptor cDNA would be tagged with epitope for monoclonal antibody and expressed in a heterologous system (eg. baculovirus-insect cell system);

2) membrane preparation (or purified receptor) incubated with various concentrations of compound for defined period;

3) membranes (or pure receptor) can be solubilized in SDS sample buffer and components separated by size on SDS-polacrylamide gels;

4) separated proteins would be transferred to nitrocellulose filter and relative positions of the tagged receptors visualized with anti-epitope antibody in immunoblot reaction;

5) monomeric and oligomeric receptor species would be identified by size and relative amounts of each species determined by densitometric scanning;

6) the ratio of monomer/oligomer species would be compared for different concentrations of the test compound.

Using the techniques of this embodiment, alternate means of separating monomeric and oligomeric receptor species by size can be used: eg. gel filtration, ultracentrifugation or others followed by antibody detection of different size forms and determination of ratio of monomeric to oligomeric species. Alternate means of labelling the receptor could entail labelling the receptor with some reporter permitting specific detection of the receptor (eg. fluorescent label specifically incorporated into the receptor protein which can be quantitated following size separation of monomeric and oligomeric species.

In yet a further embodiment, the association of monomers in oligomeric receptor complexes can be measured directly using Fluorescence Resonance Energy Transfer, involving use of two different fluorophores with distinct excitation and emission spectra, where the emission spectrum of the first fluor overlaps with excitation spectrum of the second fluor. Two separate preparations of receptor would be labelled with one or the other fluor and labelled receptor preparations would be reconstituted together in solution or in phospholipid vesicles. The mixture would then be irradiated at the excitation wavelength of the first and second fluors. Monomers would show major emission and emission wavelength for the first fluor. Oligomers would show increased emission at the emission wavelength of the second fluor due to close proximity of the two fluors and energy transfer from the first to the second fluor. The ratio of emission intensities at emission wavelengths for the first and second fluors would provide a measure of the relative amounts of monomeric (no energy transfer) and the oligomeric receptor species. Compounds which modify the ratio of monomeric and oligomeric species of the receptor will also modify the ratio of emission intensities at the two emission wavelengths and permit prediction of activity and efficacy of the compound in regulating receptor activity.

Modifications to this Fluorescence Resonance Energy Transfer method can be made by using receptors tagged with different epitopes and two corresponding monoclonal antibodies labelled with first and second fluors. In this alternative method, two receptor populations (tag 1 and tag 2) in the same preparation (by co-expression of two receptors in insect cells or mammalian lines; or by separate expression and reconstitution into single preparation) are incubated with anti-tag 1 labelled with fluor 1, and anti-tag 2 labelled with fluor 2. Monomers will not show energy transfer between fluors 1 and 2 on different receptor monomers, whereas oligomers will bring two receptor-bound antibodies into proximity and permit energy transfer, measured as an increase in emission intensity at the emission wavelength of fluor-2. Compounds would be added to the mixture and tested for their abilities to promote receptor oligomerization or dissociation of oligomers into monomers, and this information would permit prediction of compound activity and efficacy in regulating receptor function.

Finally, spectral methods can be used to measure conformational changes in proteins that can be correlated with subunit recombination [Herskovits, T.T., *Methods Enzymol.*, 11:748-755, 1967); Schmid F.X., in *Protein Structure: A Practical Approach* (Creighton, T.E.. Ed.) pp 251-286, Oxford University Press, Oxford, 1989]. Specifically, difference spectroscopy can be used to monitor the dissociation and association of subunits (Salesse, R. et al., *J. Mol. Biol.*, 95:483-496, 1975; Ingham K.C., et al.. Biochemistry, 15:1720-1726, 1976; Schmid F.X., *supra*, 1989). Reverse-phase HPLC can also be used as a method for measuring subunit recombination.

Measuring GPCR activity

The specifics of peptide-assessment assays would thus involve the following steps: Adding aqueous solution containing peptide, derivative, or peptidiomimetic compound to be tested to solution containing a GPCR preparation (tissue, cell or extract); adding agonist to the same solution; measuring the response to agonist by means of assay as described above; comparing the magnitude of the response to agonist in presence of the peptide or peptidiomimetic compound to that in absence of test molecule under otherwise identical conditions. Decrease in agonist-induced response in the presence of peptide or peptidiomimetic compound indicates antagonist activity.

Activity of GPCR-peptide can be further characterized by testing: varying concentrations of peptide with fixed concentration of peptide or peptidiomimetic compound with fixed concentration agonist (to determine the potency of the antagonist compound) and then varying the concentration of the agonist with fixed peptide concentration (to determine competitive vs. non-competitive action). Finally, measuring the effect of peptide or peptidiomimetic compound on distantly-related receptor can be performed to determine selectivity.

Activity of the GPCR-peptide or peptidiomimetic compounds can also be assessed by measuring the compound's affect on spontaneous receptor activity (i.e., basal activity in absence of added agonist). In this case, the same assay systems can be used without agonist and look for decrease in receptor activity in presence of compound.

All assays described here are familiar to those versed in the art, and described in detail in numerous scientific publications and methods manuals.

Use of Peptides as Drugs

The present invention also provides a method for treatment of G protein-coupled receptor mediated disease in patients, such as mammals, including humans, which comprises the step of administering to the patient a pharmaceutically effective amount of a compound, a pharmaceutically acceptable salt thereof, or a pharmaceutical composition as described.

The present invention also provides pharmaceutical compositions which comprise a pharmaceutically effective amount of the peptides or peptidomimetic compounds of this invention, or pharmaceutically acceptable salts thereof, and, preferably, a pharmaceutically acceptable carrier or adjuvant. Therapeutic methods of this invention comprise the step of treating patients in a pharmaceutically acceptable manner with those compounds or compositions. Such compositions may be in the form of tablets, capsules, caplets, powders, granules, lozenges, suppositories, reconstitutable powders, or liquid preparations, such as oral or sterile parenteral solutions or suspensions.

The therapeutic agents of the present invention may be administered alone or in combination with pharmaceutically acceptable carriers. The proportion of each carrier is determined by the solubility and chemical nature of the compound, the route of administration, and standard pharmaceutical practice.

In order to obtain consistency of administration, it is preferred that a composition of the invention is in the form of a unit dose. The unit dose presentation forms for oral administration may be tablets and capsules and may contain conventional excipients. For example, binding agents, such as acacia, gelatin, sorbitol, or polyvinylpyrolidone; fillers, such as lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tabletting lubricants such as magnesium stearate; disintegrants, such

as starch, polyvinylpyrrolidone, sodium starch glycollate or microcrystalline cellulose; or pharmaceutically acceptable wetting agents such as sodium lauryl sulphate.

The compounds may be injected parenterally; this being intramuscularly, intravenously, or subcutaneously. For parenternal administration, the compound may be used in the form of sterile solutions containing other solutes, for example, sufficient saline or glucose to make the solution isotonic.

The compounds may be administered orally in the form of tablets, capsules, or granules containing suitable excipients such as starch, lactose, white sugar and the like. The compounds may be administered orally in the form of solutions which may contain colouring and/or flavouring agents. The compounds may also be administered sublingually in the form of traches or lozenges in which each active ingredient is mixed with sugar or corn syrups, flavouring agents and dyes, and then dehydrated sufficiently to make the mixture suitable for pressing into solid form.

The solid oral compositions may be prepared by conventional methods of blending, filling, tabletting, or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art. The tablets may be coated according to methods well known in normal pharmaceutical practice, in particular with an enteric coating.

Oral liquid preparations may be in the form of emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may or may not contain conventional additives. For example suspending agents, such as sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethylcellulose, aluminium stearate gel, or hydrogenated edible fats; emulsifying agents, such as sorbitan monooleate or acacia; non-aqueous vehicles (which may include edible oils), such as almond oil, fractionated coconut oil, oily esters selected from the group consisting of lycerine, propylene glycol, ethylene glycol, and ethyl alcohol; preservatives, for instance methyl para-hydroxybenzoate, ethyl para-hydroxybenzoate, n-

propyl parahydroxybenzoate, or n-butyl parahydroxybenzoate of sorbic acid; and, if desired, conventional flavoring or coloring agents.

For parenteral administration, fluid unit dosage forms may be prepared by utilizing the peptide and a sterile vehicle, and, depending on the concentration employed, may be either suspended or dissolved in the vehicle. Once in solution, the compound may be injected and filter sterilized before filling a suitable vial or ampoule and subsequently sealing the carrier or storage package. Adjuvants, such as a local anaesthetic, a preservative or a buffering agent, may be dissolved in the vehicle prior to use. Stability of the pharmaceutical composition may be enhanced by freezing the composition after filling the vial and removing the water under vacuum, (e.g., freeze drying the composition). Parenteral suspensions may be prepared in substantially the same manner, except that the peptide should be suspended in the vehicle rather than being dissolved, and, further, sterilization is not achievable by filtration. The compound may be sterilized, however, by exposing it to ethylene oxide before suspending it in the sterile vehicle. A surfactant or wetting solution may be advantageously included in the composition to facilitate uniform distribution of the compound.

The pharmaceutical compositions of this invention comprise a pharmaceutically effective amount of a compound of this invention and a pharmaceutically acceptable carrier. Typically, they contain from about 0.1% to about 99% by weight, preferably from about 10% to about 60% by weight, of a compound of this invention, depending on which method of administration is employed.

Physicians will determine the dosage of the present therapeutic agents which will be most suitable. Dosages may vary with the mode of administration and the particular peptide or peptidomimtic compound chosen. In addition, the dosage may vary with the particular patient under treatment. The dosage of the compound used in the treatment will vary, depending on the seriousness of the disorder, the weight of the patient, the relative efficacy of the compound and the judgment of the treating physician. Such therapy may extend for several weeks, in an intermittent or uninterrupted manner, until the patient's symptoms are eliminated.

It is appreciated that the compounds of the present invention can be modified by one skilled in the art in such a manner as to prevent access into the central nervous system such that they can function in peripheral tissues to affect peripheral G protein coupled receptor mediated events.

To further assist in understanding the present invention, the following non-limiting examples of such peptides are provided. The following examples, of course, should not be construed as specifically limiting the present invention, variations presently known or later developed, which would be within the purview of one skilled in the art and considered to fall within the scope of the present invention as described herein.

EXAMPLES

EXAMPLE 1: DESIGN AND SYNTHESIS OF GPCR-PEPTIDE

The applicants observed that a number of chemical treatments failed to convert the dimeric species of the β_2AR to a monomeric form. These included reducing SDS-PAGE sample buffer with β-mercaptoethanol and dithiothreitol and the denaturants urea or guanidinium hydrochloride (data not shown). Other examples of SDS resistant oligomers of membrane proteins have been noted in the literature. These include glycophorin A (GpA, Harris, H.W., and Zeidel, M.L. in *The Kidney*, 5th edition, B.M. Brenner, ed., W.B. Saunders, Philadelphia, pp. 516-531, 1996), human erythrocyte band 3 (Bichet. D.G., et al., *supra*, 1994), the tailspike protein from phage P22 (Furthmayr, H. and Marchesi, V.T., *Biochemistry* 15:1137-1144, 1976), staphylococcal α-toxin (Tobkes, N., et al., *Biochemistry*, 24:1915-1920, 1985), complement membrane attack complex (Hamilton, K.K., et al., *J. Biol. Chem*, 268:3632-3638, 1993; Tschopp, J., et al., *Nature*, 298:534-538, 1982; Tschopp J., et al., *Nature* 298:534-538, 1984) and a number of porins (Schatz, G. and Butow, R.A., *Cell* 32:316-318, 1983).

In an elegant series of experiments it was demonstrated that residues located in the transmembrane domain of GpA are essential for the formation of dimers (Lemmon, M.A., et al., *Nature Struct. Biol.*

1:157-163, 1994; Borman B.J., et al., *J. Biol. Chem* **264**:4033-4037, 1989; Lemmon M.A., et al., *J. Biol. Chem*, **267**:7683-7689, 1992; Lemmon, M.A., et al., *Biochemistry* **31**:12719-12725, 1992). The transmembrane regions are believed to form a right-handed coiled coil where non-covalent helix packing (hydrophobic) interactions dominate. Based on the relative importance of specific transmembrane residues, the existence of a dimerization motif (^{75}LIXXGVXXG^{83}VXXT) was proposed for GpA. In particular, Gly^{83} was found to be essential for dimerization as substitution with either hydrophobic or larger polar residues prevented dimer formation (Lemmon, M.A., et al., *supra*, 1992). Additional glycine and leucine residues (shown in bold) were also found to be important determinants of GpA dimerization. Analysis of β_2AR transmembrane sequences revealed that leucine and glycine residues positioned with a similar spacing exist in the cytoplasmic end of the sixth transmembrane domain (TM VI): ^{272}LKTLGIIMGTFTL. Interestingly, the placement of leucines and glycines is preserved in either direction. These studies suggest that perhaps this region of the β_2AR may be involved in receptor-receptor interactions. Consistent with this hypothesis, molecular modelling has suggested that TM VI is one of the most membrane exposed of all the transmembrane segments (Baldwin, J.M. *EMBO J.*, **12**:1693-1703, 1993). Also, the leucine and glycine residues discussed above are predicted to be on the external face of the helical segment (Baldwin, supra, 1993) where they could be available for intermolecular interactions. To specifically test the idea that these residues were important for β_2AR dimerization, we synthesized a peptide corresponding to most of TM VI (residues 276-296) and assessed its ability to interfere with β_2AR dimer formation and to affect receptor-stimulated adenylyl cyclase activity.

Synthesis of peptides

Peptides were synthesized on solid-phase supports using f-moc chemistry (Merrifield, R.B., *Rec. Prog. Hormone Res.* 23:451-482, 1967; Stewart, J. and Young, J., *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Rockford, Illinois, 1984) on a BioLynx 4175 manual peptide synthesizer (LKB). Peptides were solubilized in the following buffer: 100 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA (plus a protease inhibitor cocktail consisting of 5 mg/ml leupeptin, 10 mg/ml benzamidine and 5mg/ml soybean trypsin inhibitor), 0.05% digitonin and 10% DMSO. Peptide

sequences were confirmed either by mass spectrometry or amino acid analysis. Peptides used were as follows: 1)β_2AR TM VI peptide consisting of residues 276-296; NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH, 2) a second peptide with Ala residues substituted at positions 276, 280, and 284 NH_2-AIIMATFTACWLPFFIVNIVH-COOH, 3) a peptide derived from residues 407-426 of the D2 dopamine receptor TM VII NH_2-YIIPNVASNVYGLWTFASYL-COOH, 4) a peptide derived from the C-terminal tail of the β_2AR consisting of residues 347-358 NH_2-LKAYGNGYSSNG-COOH and 5) an additional peptide unrelated to the β_2AR but of similar size as the TM VI peptide NH_2-SIQHLSTGHDHDDVDVGEQQ-COOH.

EXAMPLE II: ANALYSIS OF GPCR-PEPTIDE ACTIVITIES

To assess the effect of the different peptides on the β_2AR expressed in Sf9 and mammalian cells, the following experiments were performed. Generally, membrane preparations from mammalian or Sf9 cells infected with recombinant bacculovirus expressing human β_2AR were treated with increasing concentrations of the different peptides at room temperatures and for various times as indicated below. Specifically, membrane preparations from mammalian or Sf9 cells or affinity purified receptors derived from Sf9 cells expressing c-myc tagged β_2AR were treated at increasing concentrations of the different peptides at room temperature for various times as indicated (see results). Samples were then run on SDS-PAGE and then transferred to nitrocellulose. In some cases membrane preparations were also treated with either 10 μM timolol or 1 μM isoproterenol instead of, or in addition to the different peptides. Peptide antagonist activity was assessed by assaying adenylyl cyclase activity. In these assays, membranes were also used to determine the effect of various peptides on the ability of the β_2AR to stimulate adenylyl cyclase activity described below.

Recombinant baculoviruses

The recombinant baculoviruses encoding the c-*myc* or hemaglutinin (HA) tagged wildtype human β_2AR, the c-*myc* tagged human M2 muscarinic receptor and c-*myc* tagged D1 dopamine receptor (c-*myc* β_2AR and HA-β_2AR, c-*myc* M2-R, and c-*myc* D1-R respectively) were constructed as described

(Mouillac, B., et al., J. Biol. Cem., 267:21733-21737, 1992). Briefly, HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) and c-*myc* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) tags containing initiator methionine residues were introduced into the receptor cDNAs immediately before their initiator methionines by subcloning the corresponding double-stranded oligonucleotides. Cells were infected with recombinant baculoviruses at multiplicities of infection ranging from 3-5.

Sf9 Cell Culture

Sf9 cells were maintained at 27°C in serum-supplemented [10% fetal bovine serum (FBS) v/v] Grace's insect medium (Gibco-BRL) with gentamycin and fungizone. Cells were grown either as monolayers in T flasks or in suspension in spinner bottles supplemented with pluronic acid to prevent cell taring due to agitation. Cells were infected at log phase at a density of 1 x 10^6 cells per ml for 48 h.

Mammalian Cell Culture

CHW and LTK cell lines with and without stably transfected β_2AR were maintained as described (34). Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with L-glutamate, 10% FBS, gentamycin and fungizone. Transfected CHW cells expressed ~5 pmol receptor/mg protein while transfected LTK cells expressed 200 fmol receptor/mg protein. Stably transfected cell lines were grown in the presence of 150 ug/ml G418.

For transient expression of V2 vasopressin receptors the following procedures were followed. COS-7 cells were maintained in supplemented DMEM as described above. Genomic DNA for the V2 vasopressin receptor was isolated from nephrogenic diabetes insipidus (NDI) patients or unaffected individuals, subcloned into a construct containing a c-*myc* epitope tag and ligated into a mammalian expression vector, pBC12BI (Cullen, B.R, Meth. Enzymol., 152:684-704, 1987). Using DEAE-dextran, COS-7 cells were transiently transfected with the expression vector encoding either wildtype V2 vasopressin receptor, a truncation mutant O-11 or with vector alone for 48 hours.

Membrane Preparation

Membranes were prepared as follows and washed. Sf9 or mammalian cells were washed twice with ice-cold PBS. The cells were then disrupted by homogenization with a polytron in 10 ml of ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, 2 mM EDTA (plus a protease inhibitor cocktail consisting of 5 mg/ml leupeptin, 10 mg/ml benzamidine and 5 mg/ml soybean trypsin inhibitor). Lysates were centrifuged at 500 x *g* for 5 minutes at 4°C, the pellets homogenized as before, spun again and the supernatants were pooled. The supernatant was then centrifuged at 45,000 x *g* for 20 minutes and the pellets washed twice in the same buffer. In some cases receptors were then solubilized in 2% digitonin or 0.3% N-dodecyl-β-D-maltoside and purified by affinity chromatography on alprenolol-sepharose as or by immunoprecipitation as described below.

Affinity purification of β_2ARs

Solubilized receptors were affinity purified by alprenolol-separose chromatography as described (Mouillac, B., et al., *J. Biol. Cem.*, **267**:21733-21737, 1992; Shorr, R.G.L., et al., *J. Biol. Chem.*, **256**:5820-5826, 1981). The affinity purified preparations were concentrated using Centriprep and Centricon cartridges (Amicon) and the amount of β_2AR in each sample was determined in soluble [^{125}I]CYP radioligand binding assays as described (Mouillac, et al., 1981, *supra*). Purified receptors were desalted on Sephadex G-50 columns prior to SDS-PAGE.

Immunoprecipitation of β_2ARs

Tagged β_2ARs were immunoprecipitated with either a mouse anti-c-*myc* monoclonal antibody (9E10; Evan, G.I., et al., *Mol. Cell. Biol.*, **5**:3610-3616, 1985) or a mouse anti-hemagglutinin monoclonal antibody (12CA5; Nimar, H.L., et al., *Proc. Natl. Acad. Sci. USA*, **80**:4949-4953, 1983) as described previously (Mouillac, et al., 1981, *supra*). Removal of digitonin and concentration of the solubilized receptor was performed by dialysis using Centriprep cartridges (Amicon) against an ice-cold solution (Buffer A) containing 100 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA (plus

protease inhibitors described above) until the digitonin concentration was reduced below 0.05%. Purified 9E10 or 12CA5 antibody (1:1000 dilution) was added to the concentrate and gently agitated for 2 hours at 4°C. Anti-mouse IgG agarose (Sigma; at an 11:1 secondary to primary Ab molar ratio) and protease inhibitor cocktail were then added. The reaction was allowed to proceed overnight at 4°C with gentle agitation. The immunoprecipitate was centrifuged at 12,000 rpm in a microcentrifuge for 10 minutes at 4°C. The pellet was washed three times in buffer A and finally resuspended in 200 µL of non-reducing SDS PAGE loading buffer for 30 minutes, sonicated and centrifuged at 12,000 rpm. The supernatant was then subjected to SDS PAGE and Western blotting as described below.

Cross-linking of β_2ARs

Ten ml of Sf9 cell suspension (2 x 10^6 cells/ml) were taken 48 hours post-infection and either mock-treated with vehicle or treated with 1 mg of the membrane permeant photoactivatible cross-linking agent BASED (bis [β-(4 azidosalicylamindo) ethyl] disulphide; Pierce Chemicals) for 60 minutes at room temperature with gentle agitation. Membranes were then prepared from cells as described above and resuspended in non-reducing SDS PAGE sample buffer. Gels were subsequently immunoblotted as described below.

SDS-PAGE and Western blotting

Membrane preparations from Sf9 or mammalian cells or in some cases affinity-purified or immunoprecipitated β_2AR were prepared for non-reducing SDS-PAGE on 10% slab gels as described previously (Laemmli, U.K., *Nature*, **227**:680-686, 1970). In the case of the V2 vasopressin receptors reducing SDS-PAGE was performed. For Western blotting, gels were transferred to nitrocellulose and blotted with either the mouse anti-c-myc monoclonal antibody (9E10), the anti-hemagglutinin monoclonal antibody (12CA5) at dilutions of 1:1000 or in the case of mammalian cells expressing the β_2AR, a polyclonal rabbit anti-β_2AR antiserum raised against a peptide from the C-terminal region of the β_2AR at a dilution of 1:2000. Immunoblots against the anti-c-*myc* or anti-HA antibodies were revealed using a goat anti-mouse alkaline phosphatase-coupled second antibody (GIBCO-BRL) or

a chemiluminescent substrate for a horseradish peroxidase coupled second antibody (Renaissance, NEN Dupont). For the experiments performed using mammalian cells expressing the β_2AR western blots were developed using a chemiluminescent substrate for goat anti-rabbit coupled horseradish peroxidase antisera (Sigma). To assess total immunoreactivity of the various receptor species, blots were scanned by laser densitometry (Pharmacia-LKB Ultrascan).

Receptor quantification and adenylyl cyclase assay

Receptor number was calculated from saturation binding experiments using [^{125}I] cyanopindolol (CYP) as the radioligand (Bouvier et al., *Mol. Pharmacol.*, 267:7-19, 1994). Briefly, 10 µL of a membrane preparation in a total volume of 0.5 mL was labelled with 250 pmol of [^{125}I]-CYP which is at a near saturating concentration. Non-specific binding was defined using 10 µL alprenolol.

Adenylyl cyclase activity was assayed by the method of Salomon et al., (*Anal. Biochem.*, 58:541-548, 1974). Membranes were prepared and washed as described above. Again 10 uL of membranes (3-5 ug of protein) were used in a total volume of 50 uL. In some experiments, the peptides or the buffer used to solubilize them were added to the enzyme assay mix. Enzyme activities were determined in the presence of nM to 100 uM isoproterenol, 100 uM forskolin or 10 mM NaF. Data were calculated as pmoles cAMP produced/min/mg protein and were analyzed by least squares regression using SigmaPlot 4.17 (Jandel Scientific).

Analysis of results

Immunoblotting of c-myc epitope tagged β_2AR expressed in Sf9 cells with the anti-c-myc antibody consistently revealed the presence of molecular species corresponding to the anticipated monomeric receptor (43-50 kDa) in Sf9 cells (Mouillac, et al., 1981, *supra*) as well as higher molecular weight forms. In particular, a prominent band was detected at an apparent molecular weight corresponding to twice that of the monomer (85-95 kDa) suggesting the existence of an SDS-resistant dimeric species of the receptor. In some membrane preparations discrete bands which could represent even

higher order structures of the β_2AR can also be detected (Figure 1, lane 1). The dimer, which was readily observed in membrane preparations, was also detected in digitonin-solubilized receptors (lane 2) and following affinity purification of receptors on alprenolol-sepharose (lane 3). As shown in lanes 4 and 5, when whole cells expressing the β_2AR were treated with the membrane permeant cross-linking agent BASED, the dimer to monomer ratio as assessed by immunoblotting was increased by two-fold. This suggests that the dimer is already present before cell fractionation and that crosslinking stabilizes this form of the receptor, therefore, the dimeric species does not represent an artifact of membrane preparation or solubilization. Identical results were obtained when membranes were solubilized with 0.3% N-dodecyl-β-D-maltoside instead of digitonin (data not shown).

In order to demonstrate that the higher molecular weight species observed in this study corresponded to a specific β_2AR homodimer, we devised a differential co-immunoprecipitation strategy using c-*myc* and hemagglutinin (HA) epitope tagging. Human β_2ARs bearing either of these tags were co-expressed in Sf9 cells. The receptors were then immunoprecipitated with the anti-HA or anti-c-*myc* antibodies, subjected to SDS PAGE and blotted with one or the other antibody. In the results shown in Figure 2 the anti-HA mAb was used to blot receptors immunoprecipitated with either the anti-HA mAb or the anti-c-*myc* mAb. As seen in lane 2, blotting of the anti-HA immunoprecipitate revealed both the 45 kDa and the 90 kDa forms of the receptor. The β_2AR could also be detected by the anti-HA mAb in the c-*myc* immunoprecipitate of co-expressed receptors but the dimer then represented the predominant form (lane 1). This indicates that the two molecular species (HA-tagged and c-*myc*-tagged β_2ARs) were co-immunoprecipitated as part of a complex which is stable in SDS, consistent with the higher molecular weight form being a β_2AR homodimer. Similar but complementary results are obtained when co-expressed receptors are immunoprecipitated with either anti-c-*myc* or anti-HA antibodies and then immunoblotted with the anti-c-*myc* or anti-HA antibodies and then immunoblotted with the anti-c-*myc* antibody (data not shown). The specificity of the mAbs is illustrated by the absence of cross-reactivity in cells expressing one tagged receptor species only (Figure 2 lanes 3-6). The occurrence of intermolecular interactions appears to be receptor-specific. Indeed, although dimers of c-*myc* tagged M2 muscarinic receptor could be detected in Sf9-derived

membranes expressing this receptor (data not shown and see Debburman, S.K.. et al., *Mol. Pharmacol.*, 47:224-233, 1995) no co-immunoprecipitation with the HA-tagged β_2AR was detected when the two receptors were co-expressed (Figure 2, lanes 7,8).

V2 vasopressin receptors are also dimeric

The vasopressin receptor is critical for regulation of water retention in the kidney. Recently, several mutations of this receptor have been linked to congenital nephrogenic diabetes insipidus (NDI, Bichet, D.G., et al., *Am J. Hum. Genet.*, 55:278-286, 1994). In another approach to demonstrate GPCR dimer formation, transient expression of both wildtype and a truncated form of the V2 vasopressin receptor in COS-7 cells was studied. Both monomeric (appx. 64-69 kDa)-and dimeric (appx. 120-135 kDa) forms of the wildtype human V2 vasopressin receptor were detected when expressed in COS-7 cells (Figure 3, lane 1). A mutant form of the V2 receptor truncated in the C-terminal tail at residue 33y (O11, isolated from a patient with congenital nephrogenic diabetes insipidus (Bichet, D.G. et al., *supra,* 1994) was also capable of forming dimers when expressed in COS-7 cells (Figure 3, lane 2). Indeed, the O11 V2 receptor was detected as approx. 55-58 kD and appx. 89-100 kDa species consistent with the idea that higher molecular weight form represents a homodimer. These results confirm by a different approach that G protein-coupled receptors can form SDS-resistant dimers when expressed in mammalian cells.

Modulation of β_2AR dimerization by TM VI peptide

As shown in Figure 4a the addition of the TM VI peptide substantially reduced the amount of β_2AR dimer detected in Sf9 membranes in a time-dependent fashion (Figure 4a, lanes 1-4). In this experiment the relative amount of receptor dimer was gradually reduced from 54% at time zero to 17% after 30 minutes of treatment with TM VI peptide. When results of three such experiments were averaged, the TM VI peptide was found to reduce the relative amount of dimer by 69% after 30 minutes (Figure 4b). A control hydrophobic peptide (from transmembrane domain VII from the D2 dopamine receptor) at maximal concentration had no effect on the relative amount of dimer

detected. (Figure 4b). This does not appear to result from a non-specific hydrophobic interaction since the unrelated dopamine receptor TM VII peptide was without effect. To address the importance of the glycine and leucine residues identified above, a second control peptide corresponding to TM VI of the β_2AR with Gly 276, Gly 280 and Leu 284 replaced by alanine residues (TM VI Ala) was synthesized. Although this peptide slightly decreased the amount of dimer its effect was very modest compared with that of the TM VI peptide (Figure 4b) thus suggesting that these three residues may be a part of the interface between two receptor monomers. One mechanism which could explain the effect of the TM VI peptide is that it may interact with monomeric β_2AR thus preventing it from interacting with a second receptor monomer.

The effect of the TM VI peptide on dimer formation was also detected using purified β_2AR preparations and was shown to be dose-dependent. As seen in Figure 5a, increasing concentrations of the TM VI peptide led to a gradual reduction in the amount of dimer. This was accompanied by a concomitant increase in the level of the monomer such that the proportion of the dimer decreased from a control level of 43.1 ± 4.3% to a final level of 12.6 ± 3.2% (Figure 5a, lanes 1 - 8; Figure 5b) The D2 receptor TM VII control peptide had no effect on receptor dimerization (Figure 5a. compare lanes 9 and 10) similar to the results shown using membrane preparations (Figure 4b). We also noted a modest but reproducible upward shift in the apparent molecular weight of the monomer resulting in a widening of the band as the concentration of peptide was increased (Figure 5b, inset). This suggests that as proposed above the peptide forms a stable complex with the receptor monomer thus mimicking receptor-receptor interactions.

Functional consequences of receptor dimerization

The functional significance for receptor dimerization is suggested by the inhibitory action of the TM VI peptide on receptor-stimulated adenylyl cyclase activity. As shown in Figure 6a, the addition of TM VI peptide to membrane preparations at a concentration of 0.15 µg/µl significantly reduced isoproterenol-stimulated adenylyl cyclase activity ($p<0.05$). In contrast, neither the peptide solubilization buffer (data not shown) nor control peptides (TM VI-Ala or TM VII of the D2

dopamine receptor) had significant effects on isoproterenol-stimulated adenylyl cyclase activity. The effect of the peptide was receptor-specific as it had no effect on either NaF-mediated or forskolin-mediated adenylyl cyclase stimulation (Figure 6b). Notably, the ligand-independent basal adenylyl cyclase activity was slightly inhibited by the TM VI peptide suggesting that it may effect the spontaneous activity of the receptor as well. Indeed, spontaneous receptor activity is in large part responsible for the ligand-independent adenylyl cyclase activity observed in both Sf9 and mammalian cells expressing the β_2AR (Chiciac, P., et al., *Mol. Pharmacol.* 45:490-499, 1994). A receptor-dependent effect is also supported by the fact that the TM VI peptide was without effect on basal cyclase activity in Sf9 cells which were infected with the wildtype baculovirus (data not shown). Also consistent with a receptor-specific action of the peptide is the observation that D1 dopamine receptor-stimulated adenylyl cyclase activity was not significantly affected by the TM VI peptide (Figure 6c). As was the case for the inhibition of dimerization, the inhibitory action of the TM VI peptide on receptor-mediated adenylyl cyclase activity was dose-dependent (Figure 6c). It should be noted that the peptide IC_{50} values for the inhibition of dimer formation are very similar (2.14 ± 0.05 µM and 3.2 ± 0.04 µM, respectively) thus suggesting that receptor dimerization may be an important step in β_2AR-mediated signalling. Although our data suggest a role for dimerization in receptor activity, one cannot exclude the possibility that the effect of the TM VI peptide is not directly due to an effect on the monomer:dimer equilibrium. Still, these results clearly show that this domain of the receptor is important in modulating β_2AR signal transduction. Furthermore, the peptide represents a novel pharmacological tool for the study of receptor activity.

The effect of TM VI peptide on adenylyl cyclase stimulation does not result from a loss of receptor sites as neither the affinity or the maximum number of binding sites for ^{125}-I CYP were affected (K_D=1.8 ± 0.5 x 10^{-10} and B_{max} = 16.5 ± 2 pmol/mg protein for untreated membranes compared with K_D=4.2 ± 1.5 x 10^{-10} and B_{max} = 21.3 ± 4.5 pmol/mg protein for TM VI peptide treated membranes, n=3 for both determinations).

Effect of the TM VI peptide on GPCR in mammalian cells

In this study, β_2AR dimers were observed in CHW cells stably transfected with the receptor (Figure 8a, inset) by immunoblotting with a polyclonal anti-β_2AR antisera. Similar to our observations in Sf9 cells, the TM VI peptide also reduced the amount of β_2AR dimer detected in membane derived from CHW cells (Figure 8a, inset lane 2). This peptide also reduced basal and isoproterenol-stimulated adenylyl cyclase activity in these cells while leaving forskolin- and NaF-mediated stimulation unaffected (Figure 8a). Similar findings were also obtained with LTK$^-$ cells expressing as little as 200 fmol of β_2AR/mg protein (Figure 8b). These results taken together suggest a similar functional significance for β_2AR dimerization in mammalian cells as in Sf9 cells.

The results presented here demonstrate that both human β_2AR and V2 vasopressin receptors can form SDS-resistant homodimers. For the β_2AR, the relative amount of dimer can be altered by a peptide derived from TM VI and by receptor ligands suggesting that under basal conditions there appears to be a dynamic equilibrium between monomeric and dimeric species of receptors. The data also suggest that shifting the equilibrium away from the dimeric form of the receptor interferes with the ability of the β_2AR to productively interact with its signalling pathway.

GPCR-peptides are receptor specific

Receptor specificity is illustrated in the present examples by the observation that the M2 muscarinic receptor forms homodimers (see Debburmann, et al., *supra*, 1995 and data not shown) yet does not form heterodimers with the β_2AR (Figure 2). Similarly, the β_2AR TM VI peptide had little effect on D1 dopamine receptor-stimulated adenylyl cyclase activity (Figure 6c) or on D2 dopamine receptor dimer formation.

Application of GPCR-peptides to other GPCRs

Higher molecular weight species have been detected in both mammalian and Sf9 expression systems for many GPCRs. These include the V2 vasopressin receptor (see discussion above - this study, Figure 3), platelet activating factor receptor, metabotropic glutamate receptor, substance P receptor,

neurokinin-2 receptor, the C5a anaphylaxotoxin receptor, glucagon receptor, the dopamine D1 receptor, D2 receptor, the $5HT_{1B}$ receptor, the M2 muscarinic receptor and the M3 muscarinic receptor (see Hebert, T.E et al., *J. Biol. Chem.* accepted, 1996, and references therein). Thus, GPCR-peptides and peptidomimetic compounds could be designed for these receptors that would function to as demonstrated in these examples to selectively prevent or disrupt the functional aggregation of these receptors, thereby attenuating receptor activity.

EXAMPLE III: PEPTIDE-LEADS FOR GPCRS

Since GPCRs of the family A/rhodopsin related subfamily share a number of features common to all members of its class. One of these features is the presence of recurring patterns in their amino acid sequence. Each transmembrane domain (TM) can be characterized by a recurring pattern that is unique for that TM. The alignment of the TMs is therefore based on recurring patterns rather than on homology alone. For example, in a preferred embodiment such as presented herein, the patterns used were:

TM1: GXXXN or GN
TM2: LXXXDXXXXXXXP or LXXXDXXXXXXXXP
TM3: SXXXLXXIXXDR or SXXXLXXI XXHR
TM4: WXXXXXXXXP or WXXXXXXXXXP
TM5: FXXPXXXXXXXY
TM6: FXXCXXP
TM7: LXXXXXXXDPXXY or LXXXXXXXNPXXY

To identify a position within an alignment, rather than a position within a specific amino acid sequence, a "sequence identifier" is assigned to each position in the alignment. To obtain the "sequence identifier" for a particular residue (listed at the bottom of each column in the alignments), the "extended notation" convention is applied. This reads as follows:

3 lThis is the TM identifier.
2 lThe last two digits indicate the residue number relative to
9 lthe most conserved residue, which is number 50 by default.

This is the equivalent of, e.g., R110(3.29) in the hP2U sequence, S105(3.29) in the hAT1A sequence, or T114(3.29) in the hbeta3 sequence. A more comprehensive description of this convention can be found in: Ballesteros, J.; Weinstein, H., Meth. Neurosci., 1995, 25: 366-428. van Rhee, A.M.; Jacobson, K.A., Drug Devel. Res., 1996, 37: 1-38 (On-line as of February 28, 1995 and the last up-

date was performed on September 27, 1996).

Thrombin human P25116

TM1 102 TLFVPSVYTGVFVVSLPLNIMAIVVFILKMK 132
TM2 138 VVYMLHLATADVLFVSVLPFKISYYFSG 165
TM3 176 RFVTAAFYCNMYASILLMTVISIDR 200
TM4 215 TLGRASFTCLAIWALAIAGVVPLVLKE 241
TM5 268 AYYFSAFSAVFFFVPLIISTVCYVSIIRC 296
TM6 313 FLSAAVFCIFIICFGPTNVLLIAHYSFL 340
TM7 347 EAAYFAYLLCVCVSSISSCIDPLIYYYASSECQ 379

Human 5HT1A X13556

TM1 36 QVITSLLLGTLIFCAVLGNACVVAAIA 62
TM2 74 LIGSLAVTDLMVSVLVLPMAALYQV 98
TM3 110 DLFIALDVLCCTSSILHLCAIAL 132
TM4 152 PRALISLTWLIGFLISIPPILGWRTP 177
TM5181 DHGYTIYSTFGAFYIPLLLMLVLYGR 216
TM6 346 TLGIIMGTFILCWLPFFIVALV 366
TM7 378 TLLGAIINWLGYSNSLLNPVIYAYF 402

Human Alpha Adrenergic Receptor Subtype 2A

TM1 34 LTLVCLAGLLMLLTVFGNVLVIIAVF 59
TM2 71 FLVSLASADILVATLVIPFSLANEVM 96
TM3 107 CEIYLALDVLFCTSSIVHLCAISLDR 131
TM4 150 IKAIIITCWVISAVISFPPLISIEKK 174
TM5 193 QKWYVISCIGSFFAPCLIMILVYV 217

TM6 375 VLAVVIGVFVVCWFPFFFTYTLTAVG 400
TM7 407 LFKFFFWFGYCNSSLNPVIYTIFN 430

Human Alpha Adrenergic Receptor subtype 2C

TM1 52 AGLAAVVGFLIVFTVVGNVLVVIAV 76
TM2 89 FLVSLASADILVATLVMPFSLANELM 124
TM3 135 GVYLAL DVLFCTSSIV HLCAISLD 148
TM4 169 KATIVAVWLISAVISFPPLVSLY 191
TM5 208 TWYILSSCIGSFFAPCLIMGLVYA 231
TM6 383 LAVVMGVFVLCWFPFFFIYSLYGI 406
TM7 420 FFFWIGYCNSSLNPVIYTVFN 440

Human D3 Dopamine Receptor U25441

TM1 33 ALSYCALILAIVFGNGLLCMAVL 55
TM2 68 LVVSLAVADLLVATLVMPWVVYLEVT 94
TM3 105 VFVTLDVMMCTASILNLCAISI 127
TM4 151 VALMITAVWVLAFAVSCPLLFGF 173
TM5 187 PDFVIYSSVVSFYLPFGVTVLVYA 210
TM6 331 MVAIVLGTFIVCWLPFFLTHVLNT 354
TM7 368 ATTWLGYVNSALNPVIYTTFNI 389

Histamine H2 human P25021

TM 118 KITITVVLAVLILITVAGNVVVCLAVGLNRR 48
TM2 54 NCFIVSLAITDLLLGLLVLPFSAIYQLS 81
TM3 92 NIYTSLDVMLCTASILNLFMISLDR 116
TM4 131 TPVRVAISLVLIWVISITLSFLSIHLG 157

TM5 180 EVYGLVDGLVTFYLPLLIMCITYYRIFKV 208
TM6 234 VTLAAVMGAFIICWFPYFTAFVYRGLRG 261
TM7 264 AINEVLEAIVLWLGYANSALNPILYAALNRDFR 296

Adenosine A1 human P30542

TM1 9 QAAYIGIEVLIALVSVPGNVLVIWAVKVNQA 39
TM2 45 FCFIVSLAVADVAVGALVIPLAILINIG 72
TM3 81 LMVACPVLILTQSSILALLAIAVDR 105
TM4 120 TPRRAAVAIAGCWILSFVVGLTPMFGW 146
TM5 178 EYMVYFNFFVWVLPPLLLMVLIYLEVFYL 206
TM6 234 KSLALILFLFALSWLPLHILNCITLFCP 261
TM7 264 HKPSILTYIAIFLTHGNSAMNPIVYAFRIQKFR 296

Adenosine A2a human P29274

TM1 6 SSVYITVELAIAVLAILGNVLVCWAVWLNSN 36
TM2 42 NYFVVSLAAADIAVGVLAIPFAITISTG 69
TM3 78 LFIACFVLVLTQSSIFSLLAIAIDR 102
TM4 117 TGTRAKGIIAICWVLSFAIGLTPMLGW 143
TM5 175 NYMVYFNFFACVLVPLLLMLGVYLRIFLA 203
TM6 233 KSLAIIVGLFALCWLPLHIINCFTFFCP 260
TM7 264 HAPLWLMYLAIVLSHTNSVVNPFIYAYRIREFR 296

Dopamine Receptor subtype 2 (long form) M29066

TM1 38 LTLLIAVIVFGNVVCMAVS 60
TM2 72 LIVSLAVADLLVATLVMPWVV 92
TM3 109 IFVTLDVMMCTASILNLCAISI 130

TM4 152 VTVMISIVWVLSFTISCPLLFGL 174
TM5 188 PAFVVYSSIVSFYV PFIVTLLVYI 210
TM6 373 QMLAIVLGVFIICWLPFFITHILNI 397
TM7 406 VLYSAFTWLGYVNSAVNPIIYTTF 429

CC CR1 human P32246

TM1 34 AQLLPPLYSLVFVIGLVGNILVVLVLVQYKR 64
TM2 70 SIYLLNLAISDLLFLFTLPFWIDYKLKD 97
TM3 107 KILSGFYYTGLYSEIFFIILLTIDR 131
TM4 146 TVTFGVITSIIIWALAILASMPGLYFS 172
TM5 197 LFQALKLNLFGLVLPLLVMIICYTGIIKI 225
TM6 239 RLIFVIMIIFFLFWTPYNLTILISVFQD 266
TM7 277 RHLDLAVQVTEVIAYTHCCVNPVIYAFVGERFR 309

Platelet Activating Factor human P25105

TM1 115 YTLFPIVYSIIFVLGVIANGYVLWVFARLYP 45
TM2 53 KIFMVNLTMADMLFLITLPLWIVYYQNQ 80
TM3 91 NVAGCLFFINTYCSVAFLGVITYNR 115
TM4 129 NTRKRGILSLVIWVAIVGAASYFLILD 155
TM5 184 VLIIHIFIVFSFFLVFLIILFCNLVIIRT 212
TM6 232 WMVCTVLAVFIICFVPHHVVQLPWTLA 259
TM7 269 QAINDAHQVTLCLLSTNCVLDPVIYCFLTKKFR 301

Beta3 Adrenergic Receptor human P13945

TM 137 AALAGALLALAVLATVGGNLLVIVAIAWTPR 67
TM2 73 NVFVTSLAAADLVMGLLVVPPAATLALT 100

TM3 111 ELWTSVDVLCVTASIETLCALAVDR 135
TM4 150 TKRCARTAVVLVWVVSAAVSFAPIMSQ 176
TM5 202 MPYVLLSSSVSFYLPLLVMLFVYARVFVV 230
TM6 292 CTLGLIMGTFTLCWLPFFLANVLRALGG 319
TM7 322 LVPGPAFLALNWLGYANSAFNPLIYCRSPDFRS 354

P2U human L14751

TM1 33 YVLLPVSYGVVCVLGLCLNAVGLYIFLCRLK 63
TM2 69 TTYMFHLAVSDALYAASLPLLVYYYARG 96
TM3 107 KLVRFLFYTNLYCSILFLTCISVHR 131
TM4 146 RARYARRVAGAVWVLVLACQAPVLYFV 172
TM5 195 VAYSSVMLGLLFAVPFAVILVCYVLMARR 223
TM6 244 RTIAVVLAVFALCFLPFHVTRTLYYSFR 271
TM7 281 NAINMAYKVTRPLASANSCLDPVLYFLAGQRLV 313

Chemokine CC CR5

TM1 18 IAARLLPPLYSLVFIFGFVGNMLVILILI 57
TM2 67 IYLLNLAISDLFFLLTVPFWAHYLAAQ 93
TM3 103 LLTGLYFIGFFSGIFFIILLT 124
TM4 142 TVTFGVVTSVITWVVAVFASLPGIIFTRSQ 170
TM 193 FQTLKIVILGLVLPLLVMVICYSGILKTLLF 223
TM6 234 LIFTIMIVYFLFWAPYNIVLLLNTFQ 259
TM7 275 QAMQVTETLGMTHCCINPIIYAFV 298

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Consequently, such

changes and modifications are properly, equitably, and "intended" to be, within the full range of equivalence of the following claims.

WE CLAIM:

1. A peptide or peptide-lead modeled on a transmembrane domain of a G protein-coupled receptor characterized by a recurring pattern that is unique for that transmembrane domain, wherein said peptide or peptide-lead is characterized by the ability to selectively affect oligomerization or the G protein-coupled receptor from which it was designed

2. A peptide, analog, fragment or derivative thereof which is characterized as being:
(a) 15 - 30 amino acid residues in length;
(b) significantly homologous to a transmembrane domain of a G protein-coupled receptor;
(c) possessing the ability to alter the ratio of monomeric-to-multimeric G protein-coupled receptors of the parent G protein-coupled receptor and possibly closely related receptors; and
(d) demonstrating the inability to significantly alter the ratio of monomeric-to-multimeric G protein-coupled receptors for other types of receptors.

3. A peptidomimetic compound modeled on a peptide, analog, fragment or derivative thereof which is characterized as being:
(a) 15 - 30 amino acid residues in length;
(b) significantly homologous to a transmembrane domain of a G protein-coupled receptor;
(c) possessing the ability to alter the ratio of monomeric-to-multimeric G protein-coupled receptors of the parent G protein-coupled receptor and possibly closely related receptors; and
(d) demonstrating the inability to significantly alter the ratio of monomeric-to-multimeric G protein-coupled receptors for other types of receptors.

4. A composition comprising one or more compounds of claim 1 in a pharmaceutically acceptable carrier.

5. A composition comprising one or more compounds of claim 2 in a pharmaceutically acceptable carrier.

6. A composition comprising one or more compounds of claim 3 in a pharmaceutically acceptable carrier.

7. A peptide or peptide-lead modeled on a transmembrane domain of a G protein-coupled receptor characterized by a recurring pattern that is unique for that transmembrane domain, wherein said peptide or peptide-lead is characterized by the ability to selectively affect the ratio of monomer-to-multimer.

8. A method for inhibiting G protein-coupled receptor mediated processes comprising administering a pharmaceutically effective amount of a composition according to claim 4 effective to modulate the activity of said G protein-coupled receptor binding its agonist or antagonist, and a pharmaceutically acceptable carrier.

9. A method of identifying novel peptide modulators of G protein-coupled receptors comprising the steps:

(a) identifying and defining the peptide-lead characterized by a recurring pattern that is unique for that transmembrane domain,

(b) testing the ability of the peptide-lead to significantly alter the ratio of monomeric-to-multimeric G protein-coupled receptors of the parent G protein-coupled receptor and possibly closely related receptors; and

(c) testing the inability of the peptide-lead to significantly alter the ratio of monomeric-to-multimeric G protein-coupled receptors for other types of receptors.

10. A DNA sequence for a compound as in claim 1.

11. A DNA sequence for a compound as in claim 2.

12. An expression vector comprising the DNA sequence of claim 11.

13. An expression vector comprising the DNA sequence of claim 12.

14. A method of making a peptide that is significantly homologous to a transmembrane domain of a G protein-coupled receptor that selectively modulates the activity of a G protein-coupled receptor comprising the steps producing a biologically active peptide demonstrating the ability to selectively modulate G protein-coupled receptor activity comprising:

a) constructing an expression vector containing a DNA sequence encoding the biologically active peptide;

b) transforming a bacterial host cell with the vector;

c) and culturing the transformed host cell such that the peptide is expressed; and

d) recovering the biologically active peptide.

15. A method of treating a living being with a peptide or peptidomimetic modulator of G protein-coupled receptor activity comprising the steps:

a) preparing a compound comprising a peptide, fragment, analog or derivative of a transmembrane domain sequence of a protein-coupled receptor, said compound having the ability to alter the ratio of monomeric-to-multimeric G protein-coupled receptors of the parent G protein-coupled receptor and possibly closely related receptors and demonstrating the inability to significantly alter the ratio of monomeric-to-multimeric G protein-coupled receptors for other types of receptors;

b) combining the synthetic peptide with a delivery vehicle; and

c) administering to a living being a G protein-coupled receptor modulating amount of the agent.

16. A method for preventing or treating neurological disorders involving G protein-coupled receptors which comprises administering to a living being in need thereof an amount of the composition of claim 1 effective to prevent or treat said disorder.

17. A method for preventing or treating genetic disorders involving G protein-coupled receptors which comprises administering to a living being in need thereof an amount of the composition of claim 1 effective to prevent or treat said disorder.

18. A method for preventing or treating disease involving G protein-coupled receptors which comprises administering to a living being in need thereof an amount of the composition of claim 1 effective to prevent or treat said disease.

19. A peptide or peptide-lead as in claim 1 that is GIIMGTFTLCWLPFFIVNIV.

20. A peptide or peptide-lead as in claim 1 whose sequence is chosen from one or more of the transmembrane domain sequences:

Thrombin human P25116

TM1 102 TLFVPSVYTGVFVVSLPLNIMAIVVFILKMK 132
TM2 138 VVYMLHLATADVLFVSVLPFKISYYFSG 165
TM3 176 RFVTAAFYCNMYASILLMTVISIDR 200
TM4 215 TLGRASFTCLAIWALAIAGVVPLVLKE 241
TM5 268 AYYFSAFSAVFFFVPLIISTVCYVSIIRC 296
TM6 313 FLSAAVFCIFIICFGPTNVLLIAHYSFL 340
TM7 347 EAAYFAYLLCVCVSSISSCIDPLIYYYASSECQ 379

Human 5HT1A X13556

TM1 36 QVITSLLLGTLIFCAVLGNACVVAAIA 62
TM2 74 LIGSLAVTDLMVSVLVLPMAALYQV 98
TM3 110 DLFIALDVLCCTSSILHLCAIAL 132
TM4 152 PRALISLTWLIGFLISIPPILGWRTP 177
TM5181 DHGYTIYSTFGAFYIPLLLMLVLYGR 216
TM6 346 TLGIIMGTFILCWLPFFIVALV 366
TM7 378 TLLGAIINWLGYSNSLLNPVIYAYF 402

Human Alpha Adrenergic Receptor Subtype 2A

TM1 34 LTLVCLAGLLMLLTVFGNVLVIIAVF 59
TM2 71 FLVSLASADILVATLVIPFSLANEVM 96
TM3 107 CEIYLALDVLFCTSSIVHLCAISLDR 131
TM4 150 IKAIIITCWVISAVISFPPLISIEKK 174
TM5 193 QKWYVISCIGSFFAPCLIMILVYV 217
TM6 375 VLAVVIGVFVVCWFPFFFTYTLTAVG 400
TM7 407 LFKFFFWFGYCNSSLNPVIYTIFN 430

Human Alpha Adrenergic Receptor subtype 2C

TM1 52 AGLAAVVGFLIVFTVVGNVLVVIAV 76
TM2 89 FLVSLASADILVATLVMPFSLANELM 124
TM3 135 GVYLAL DVLFCTSSIV HLCAISLD 148
TM4 169 KATIVAVWLISAVISFPPLVSLY 191
TM5 208 TWYILSSCIGSFFAPCLIMGLVYA 231
TM6 383 LAVVMGVFVLCWFPFFFIYSLYGI 406
TM7 420 FFFWIGYCNSSLNPVIYTVFN 440

Human D3 Dopamine Receptor U25441

TM1 33 ALSYCALILAIVFGNGLLCMAVL 55
TM2 68 LVVSLAVADLLVATLVMPWVVYLEVT 94
TM3 105 VFVTLDVMMCTASILNLCAISI 127
TM4 151 VALMITAVWVLAFAVSCPLLFGF 173
TM5 187 PDFVIYSSVVSFYLPFGVTVLVYA 210
TM6 331 MVAIVLGTFIVCWLPFFLTHVLNT 354
TM7 368 ATTWLGYVNSALNPVIYTTFNI 389

Histamine H2 human P25021

TM 118 KITITVVLAVLILITVAGNVVVCLAVGLNRR 48
TM2 54 NCFIVSLAITDLLLGLLVLPFSAIYQLS 81
TM3 92 NIYTSLDVMLCTASILNLFMISLDR 116
TM4 131 TPVRVAISLVLIWVISITLSFLSIHLG 157
TM5 180 EVYGLVDGLVTFYLPLLIMCITYYRIFKV 208
TM6 234 VTLAAVMGAFIICWFPYFTAFVYRGLRG 261
TM7 264 AINEVLEAIVLWLGYANSALNPILYAALNRDFR 296

Adenosine A1 human P30542

TM1 9 QAAYIGIEVLIALVSVPGNVLVIWAVKVNQA 39
TM2 45 FCFIVSLAVADVAVGALVIPLAILINIG 72
TM3 81 LMVACPVLILTQSSILALLAIAVDR 105
TM4 120 TPRRAAVAIAGCWILSFVVGLTPMFGW 146

TM5 178 EYMVYFNFFVWVLPPLLLMVLIYLEVFYL 206

TM6 234 KSLALILFLFALSWLPLHILNCITLFCP 261

TM7 264 HKPSILTYIAIFLTHGNSAMNPIVYAFRIQKFR 296

Adenosine A2a human P29274

TM1 6 SSVYITVELAIAVLAILGNVLVCWAVWLNSN 36

TM2 42 NYFVVSLAAADIAVGVLAIPFAITISTG 69

TM3 78 LFIACFVLVLTQSSIFSLLAIAIDR 102

TM4 117 TGTRAKGIIAICWVLSFAIGLTPMLGW 143

TM5 175 NYMVYFNFFACVLVPLLLMLGVYLRIFLA 203

TM6 233 KSLAIIVGLFALCWLPLHIINCFTFFCP 260

TM7 264 HAPLWLMYLAIVLSHTNSVVNPFIYAYRIREFR 296

Dopamine Receptor subtype 2 (long form) M29066

TM1 38 LTLLIAVIVFGNVVCMAVS 60

TM2 72 LIVSLAVADLLVATLVMPWVV 92

TM3 109 IFVTLDVMMCTASILNLCAISI 130

TM4 152 VTVMISIVWVLSFTISCPLLFGL 174

TM5 188 PAFVVYSSIVSFYV PFIVTLLVYI 210

TM6 373 QMLAIVLGVFIICWLPFFITHILNI 397

TM7 406 VLYSAFTWLGYVNSAVNPIIYTTF 429

CC CR1 human P32246

TM1 34 AQLLPPLYSLVFVIGLVGNILVVLVLVQYKR 64

TM2 70 SIYLLNLAISDLLFLFTLPFWIDYKLKD 97

TM3 107 KILSGFYYTGLYSEIFFIILLTIDR 131

TM4 146 TVTFGVITSIIIWALAILASMPGLYFS 172

TM5 197 LFQALKLNLFGLVLPLLVMIICYTGIIKI 225

TM6 239 RLIFVIMIIFFLFWTPYNLTILISVFQD 266

TM7 277 RHLDLAVQVTEVIAYTHCCVNPVIYAFVGERFR 309

Platelet Activating Factor human P25105

TM1 115 YTLFPIVYSIIFVLGVIANGYVLWVFARLYP 45

TM2 53 KIFMVNLTMADMLFLITLPLWIVYYQNQ 80

TM3 91 NVAGCLFFINTYCSVAFLGVITYNR 115

TM4 129 NTRKRGILSLVIWVAIVGAASYFLILD 155

TM5 184 VLIIHIFIVFSFFLVFLIILFCNLVIIRT 212

TM6 232 WMVCTVLAVFIICFVPHHVVQLPWTLA 259

TM7 269 QAINDAHQVTLCLLSTNCVLDPVIYCFLTKKFR 301

Beta3 Adrenergic Receptor human P13945

TM 137 AALAGALLALAVLATVGGNLLVIVAIAWTPR 67

TM2 73 NVFVTSLAAADLVMGLLVVPPAATLALT 100

TM3 111 ELWTSVDVLCVTASIETLCALAVDR 135

TM4 150 TKRCARTAVVLVWVVSAAVSFAPIMSQ 176

TM5 202 MPYVLLSSSVSFYLPLLVMLFVYARVFVV 230

TM6 292 CTLGLIMGTFTLCWLPFFLANVLRALGG 319

TM7 322 LVPGPAFLALNWLGYANSAFNPLIYCRSPDFRS 354

P2U human L14751

TM1 33 YVLLPVSYGVVCVLGLCLNAVGLYIFLCRLK 63

TM2 69 TTYMFHLAVSDALYAASLPLLVYYYARG 96

TM3 107 KLVRFLFYTNLYCSILFLTCISVHR 131

TM4 146 RARYARRVAGAVWVLVLACQAPVLYFV 172

TM5 195 VAYSSVMLGLLFAVPFAVILVCYVLMARR 223

TM6 244 RTIAVVLAVFALCFLPFHVTRTLYYSFR 271

TM7 281 NAINMAYKVTRPLASANSCLDPVLYFLAGQRLV 313

Chemokine CC CR5

TM1 18 IAARLLPPLYSLVFIFGFVGNMLVILILI 57

TM2 67 IYLLNLAISDLFFLLTVPFWAHYLAAQ 93

TM3 103 LLTGLYFIGFFSGIFFIILLT 124

TM4 142 TVTFGVVTSVITWVVAVFASLPGIIFTRSQ 170

TM 193 FQTLKIVILGLVLPLLVMVICYSGILKTLLF 223

TM6 234 LIFTIMIVYFLFWAPYNIVLLLNTFQ 259

TM7 275 QAMQVTETLGMTHCCINPIIYAFV 298

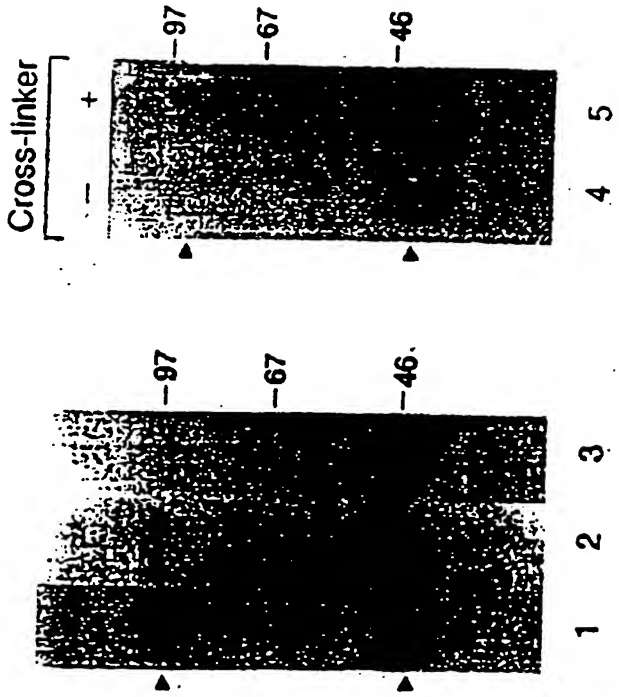

FIGURE 1

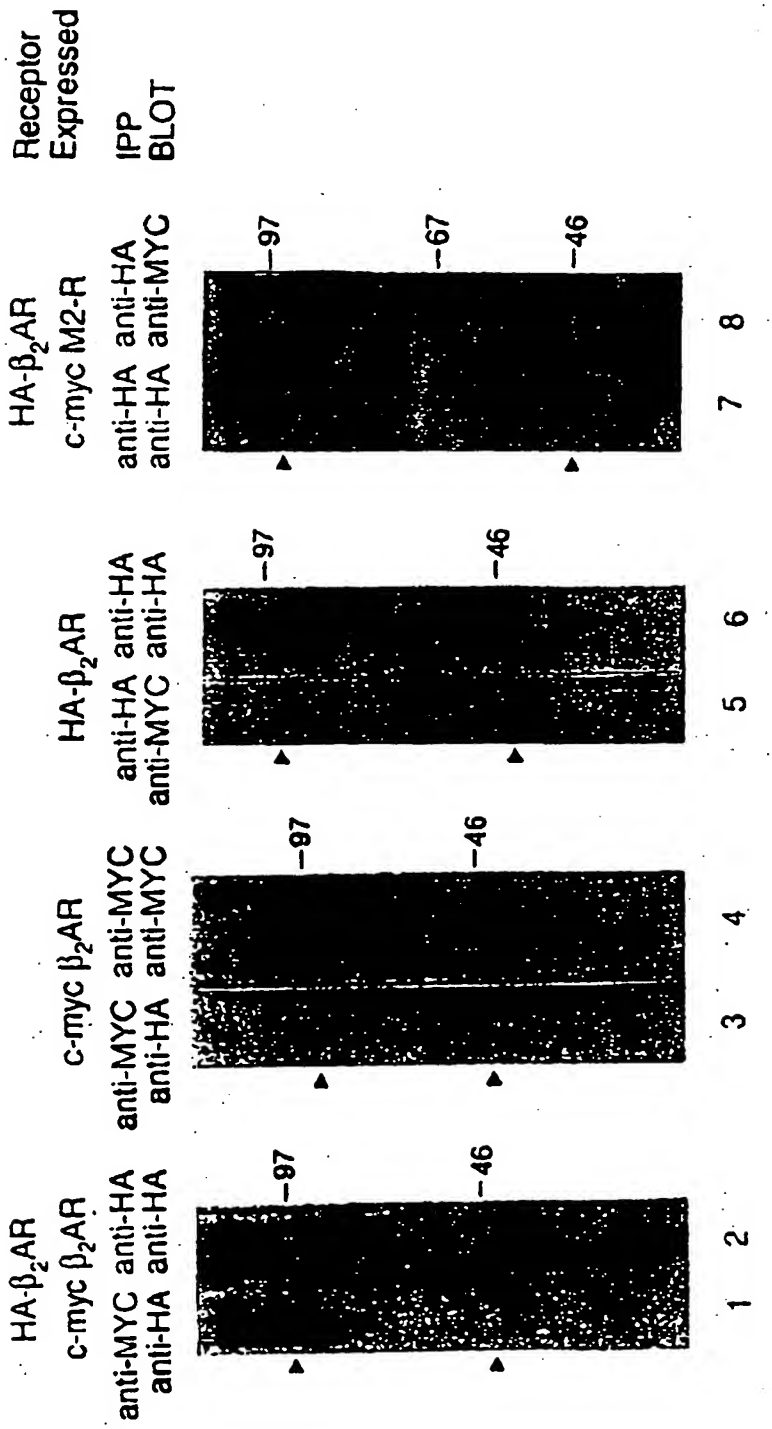

FIGURE 2

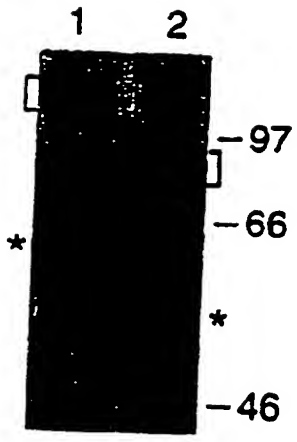

FIGURE 3

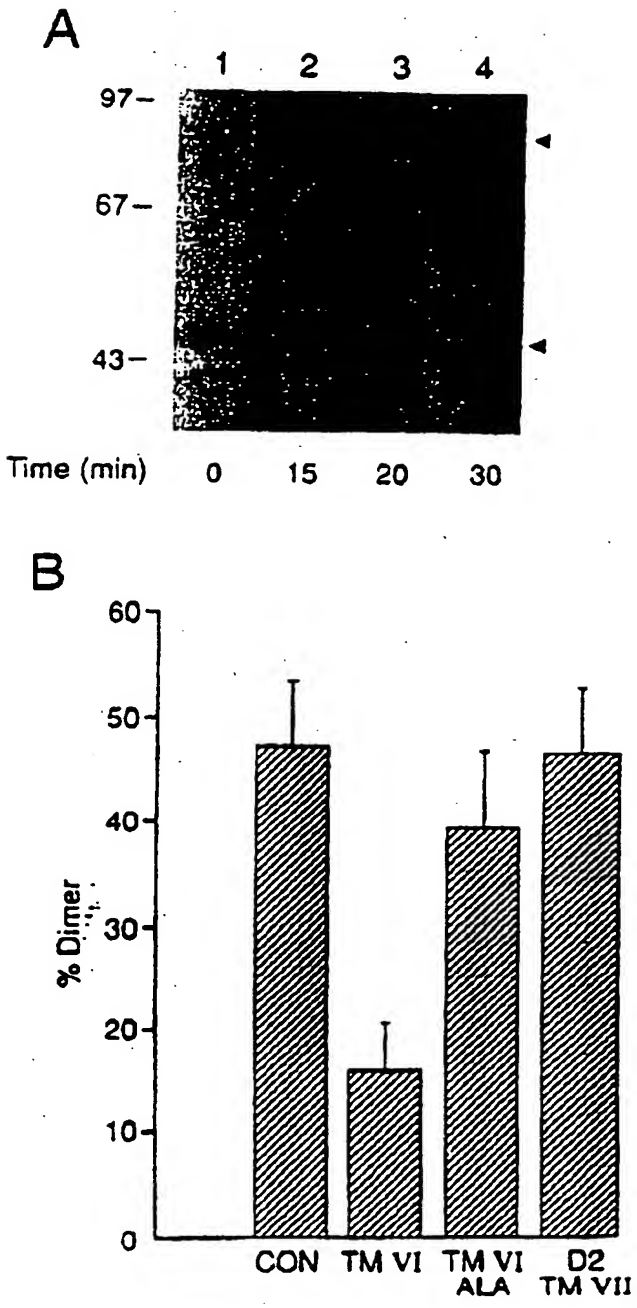
A
1
2
3
4
97–
67–
43–
Time (min)
0
15
20
30
B
60
50
40
30
20
10
0
% Dimer
CON
TM VI
TM VI ALA
D2 TM VII

FIGURE 4

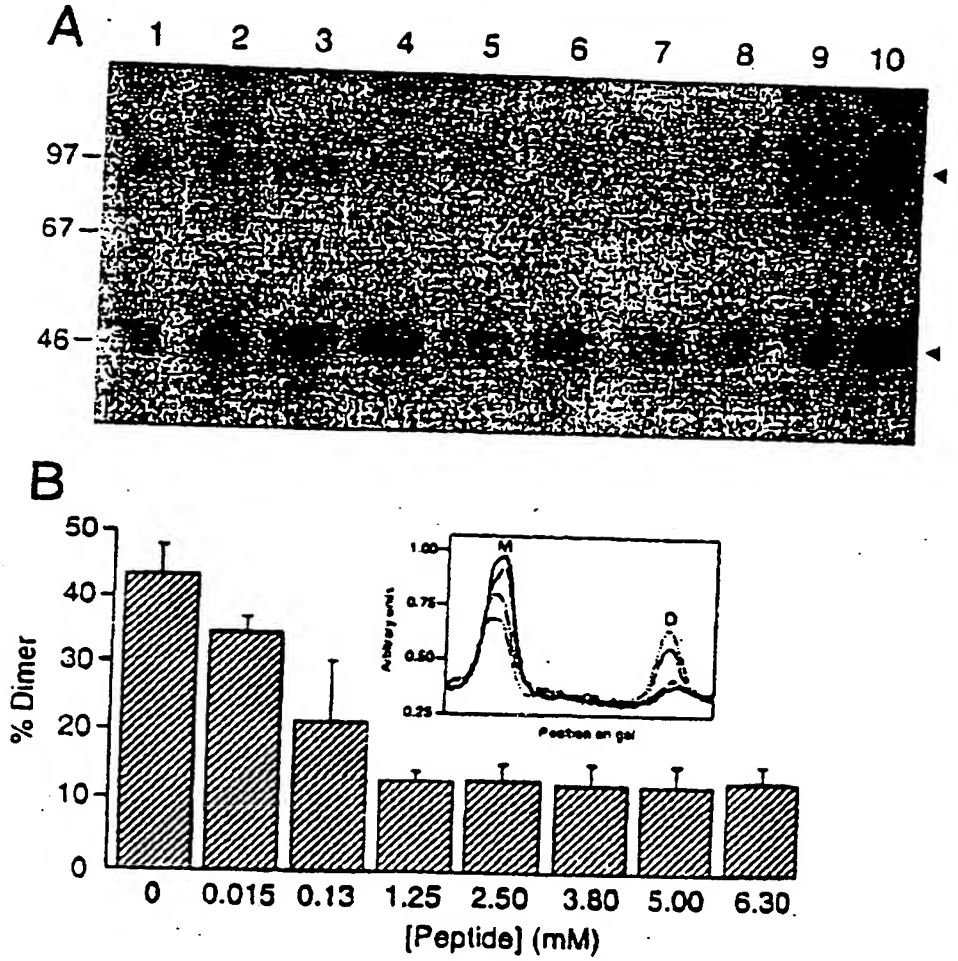
A
1 2 3 4 5 6 7 8 9 10
97
67
46
B
% Dimer
50
40
30
20
10
0
0 0.015 0.13 1.25 2.50 3.80 5.00 6.30
[Peptide] (mM)
M
D

FIGURE 5

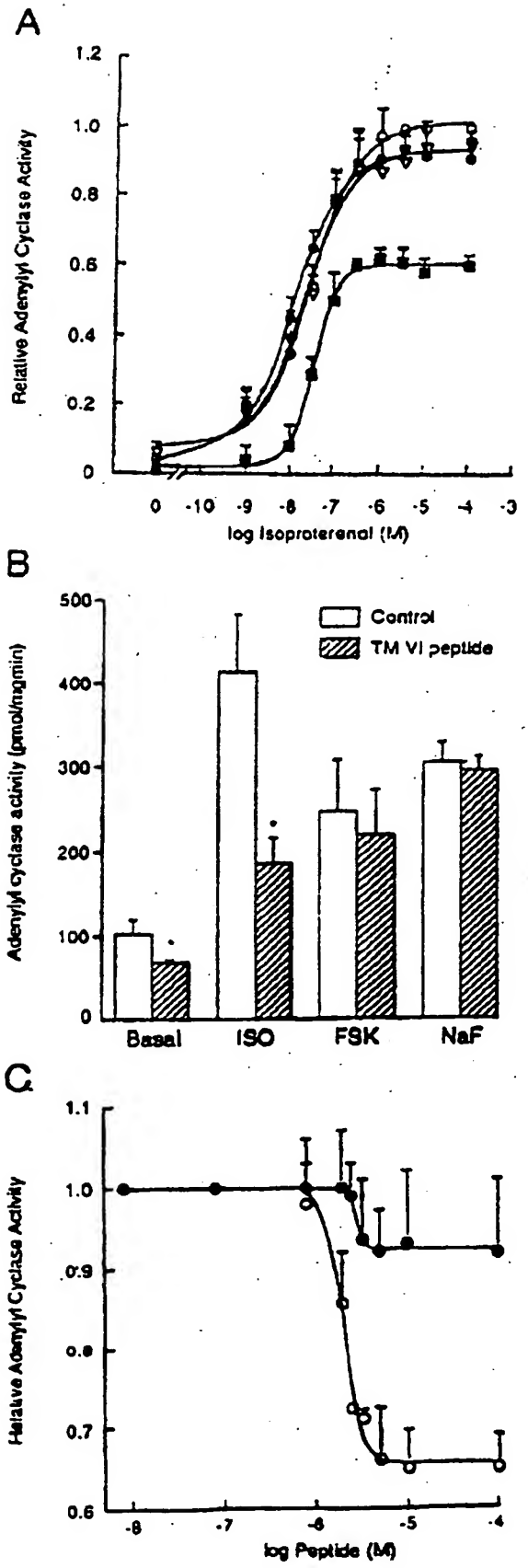

FIGURE 6

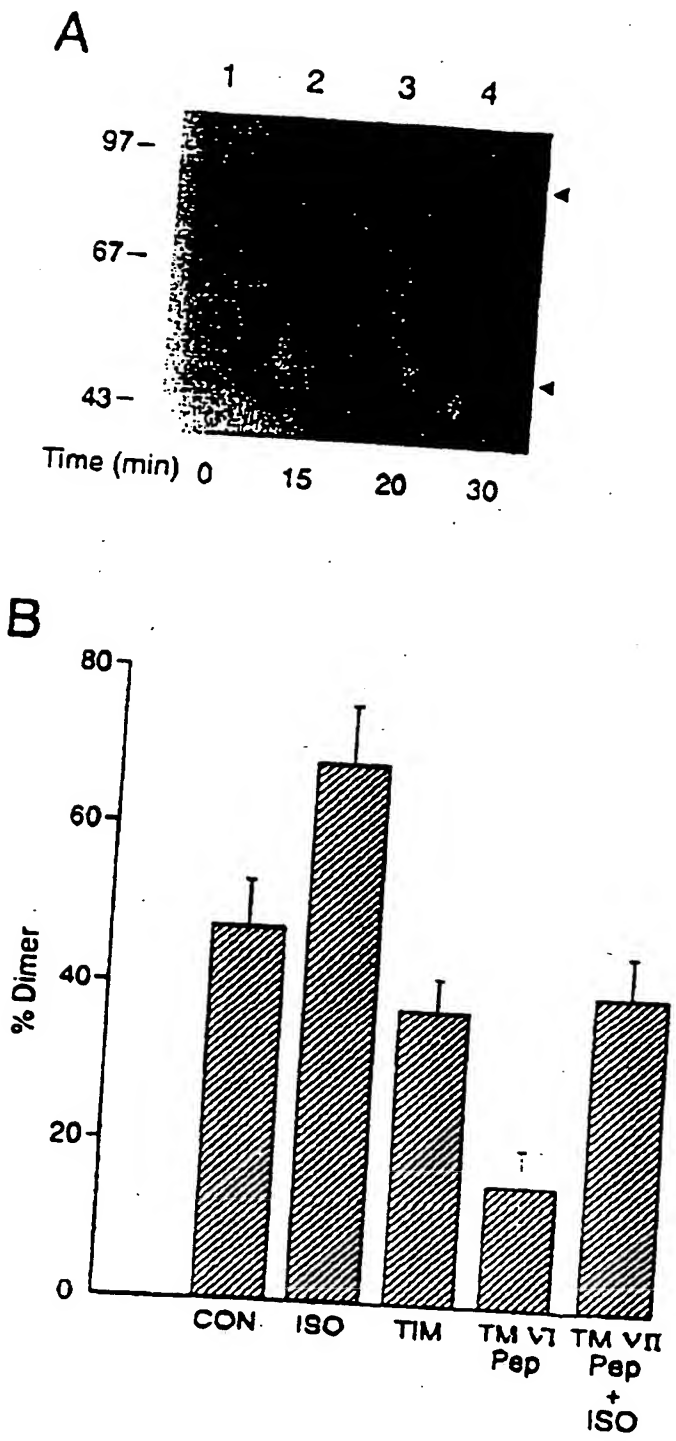

FIGURE 7

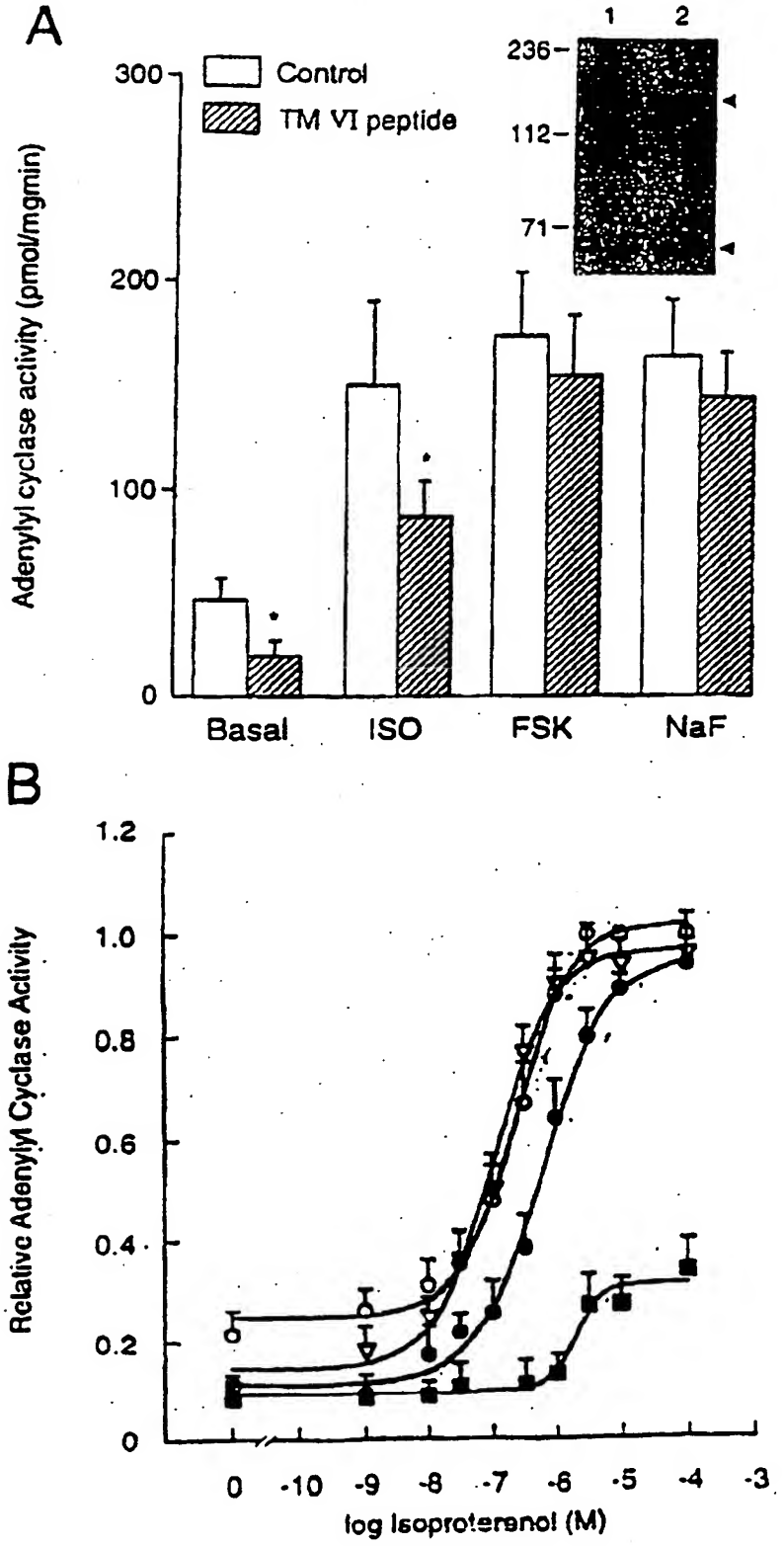

FIGURE 8

FIGURE 9 List of receptors with and without GGL motif and accompanying reference sources.

β2AR TM VI	LKTL**G**IIM**G**TFTI**L**CWLPFFIVH		
	G	G	L
Adrenergic receptors			
α1A	A	+	+
α1B	+	+	+
α1C	+	+	+
α2A	A	+	V
α2B	A	+	+
α2C	A	+	+
β1	+	+	+
β2	+	+	+
β3	+	+	+
Dopamine receptors			
D1	–	+	+
D2	–	–	–
D4C	–	–	–
D5	–	+	+
Vasopressin receptors			
V2	–	–	–
Muscarinic receptors			
M1	–	–	–
M2	–	–	–
M3	–	–	–
M4	–	–	–
M5	–	–	–

Serotonin receptors			
HT1b	+	+	V
HT1c	+	F	+
HT1d	+	+	I
HT1e	+	+	+
HT1f	+	+	I
HT2	+	F	I
HT7	+	+	V
Thyroptropin receptor	-	-	-

1) Intra- and Intermolecular interactions in GPCRs studied using peptides or mini-genes

Hawes et al., (1994) JBC 269:15776 Inhibition of GPCR signalling by expression of cytoplasmic domains of the receptor.

Luttrell et al., (1993) Science 259:1453- same group, same subject

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Maggio, R., et al (1993) Proc. Natl. Acad. Sci. USA 90:3103

Liu et al., (1995) JBC 270:19532 Mutational analysis of the relative orientation of transmembrane helices I and VII in GPCRs- these last two papers are from the Wess group

Sealfon et al. (1995) JBC 270:16683 Related contribution of specific helix 2 and 7 residues to conformational activation of the serotonin 5-HT_{2A} receptor.

Zhou et al., (1994) Mol. Pharmacol. 45:165 A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotrophin-releasing hormone receptor.

Mizobe et al., (1996) JBC 271:2387 Arrangement of transmembrane domains in adrenergic receptors.

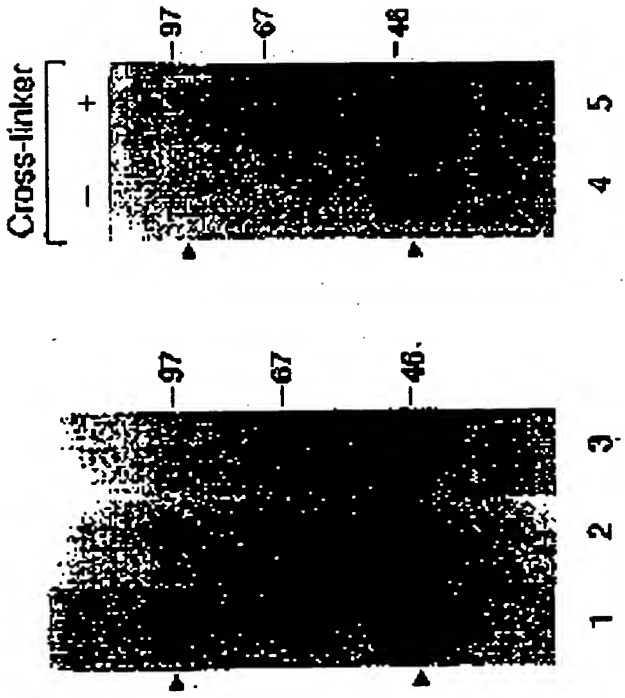


FIGURE 1

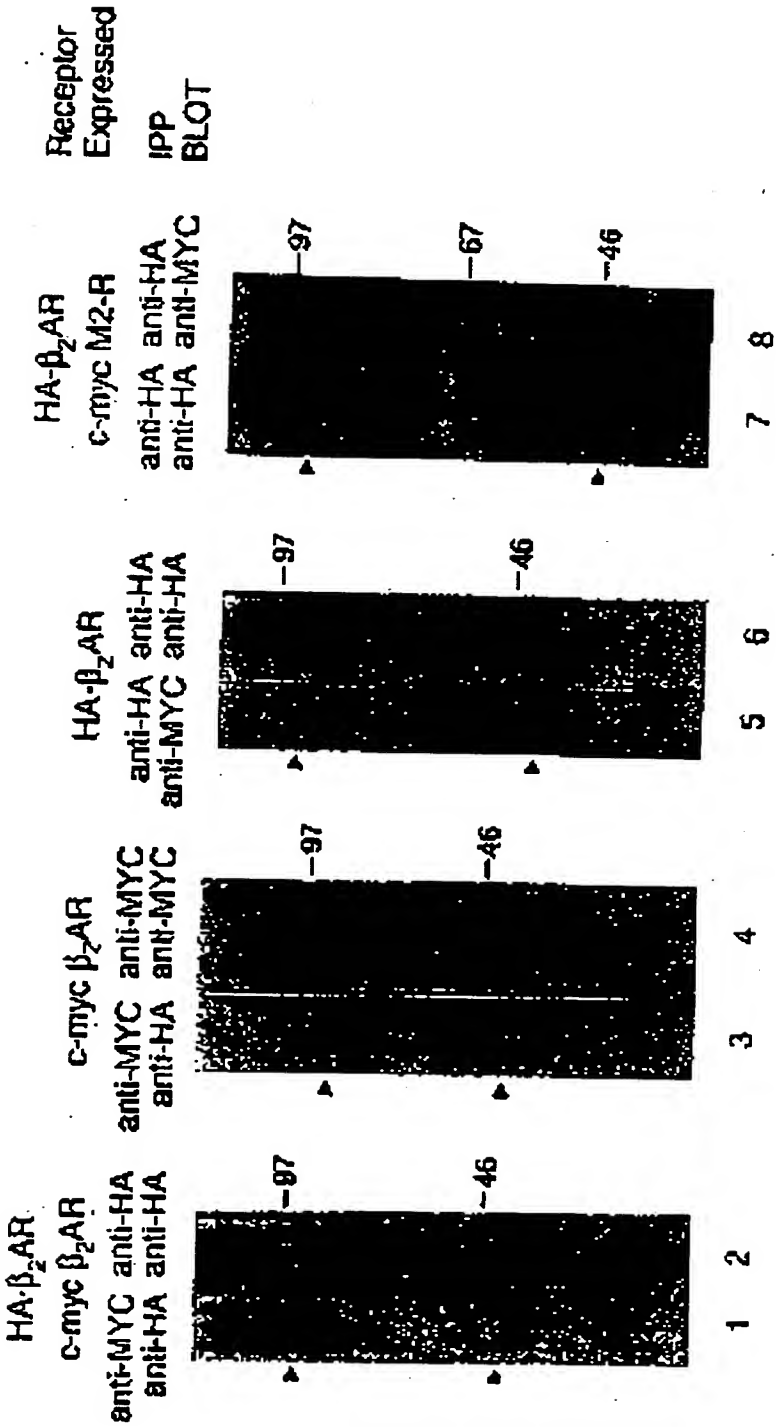
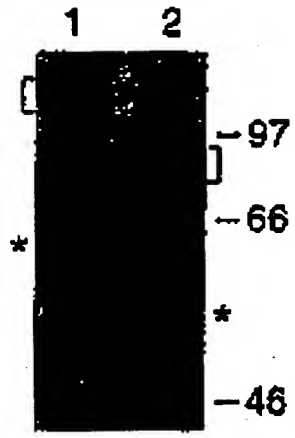


FIGURE 2



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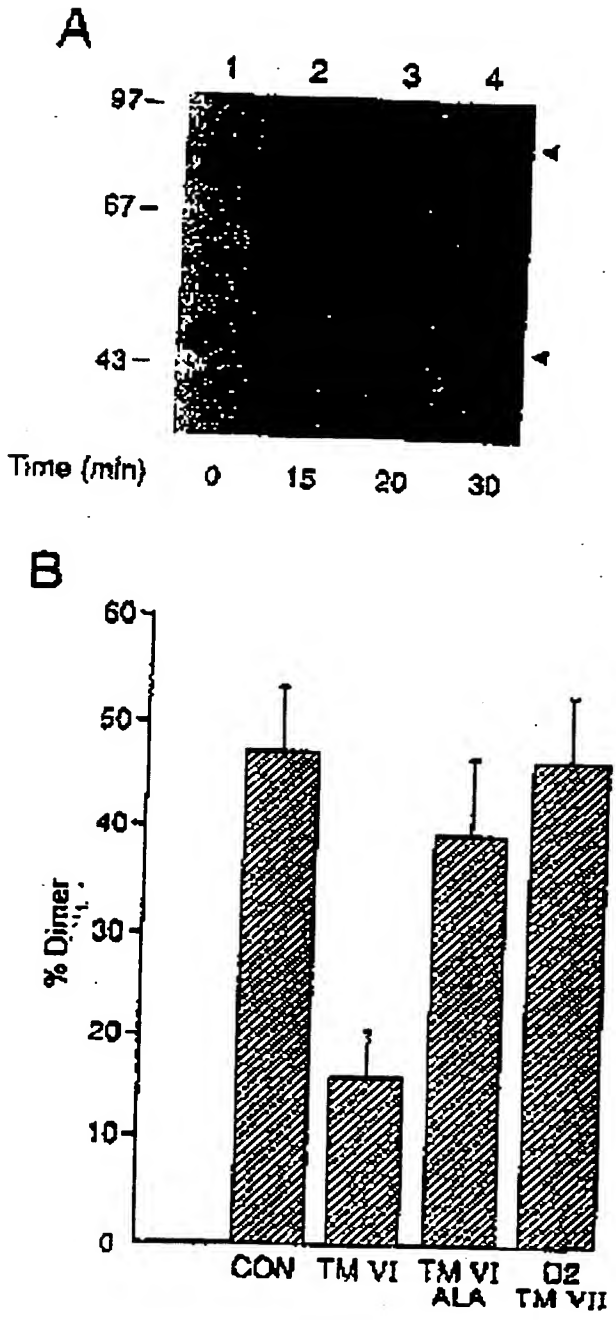

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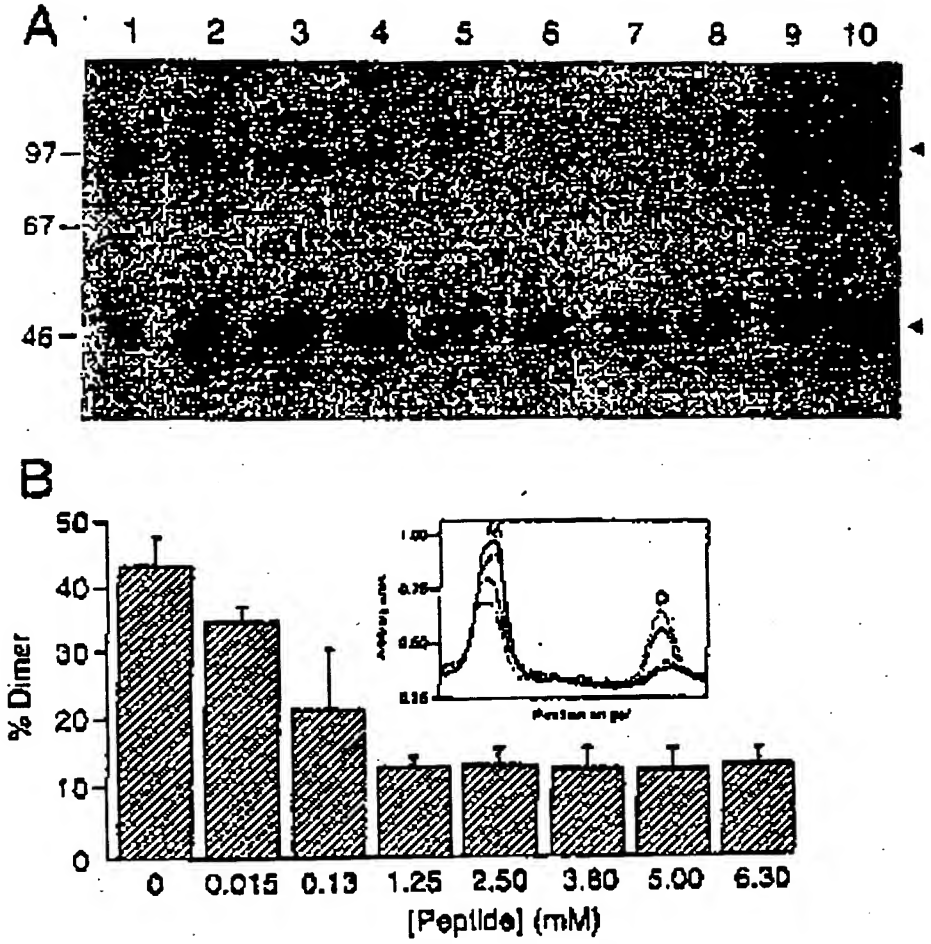
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1 2 3 4 5 6 7 8 9 10
97
67
46
B
% Dimer
50
40
30
20
10
0
0 0.015 0.13 1.25 2.50 3.80 5.00 6.30
[Peptide] (mM)

FIGURE 5

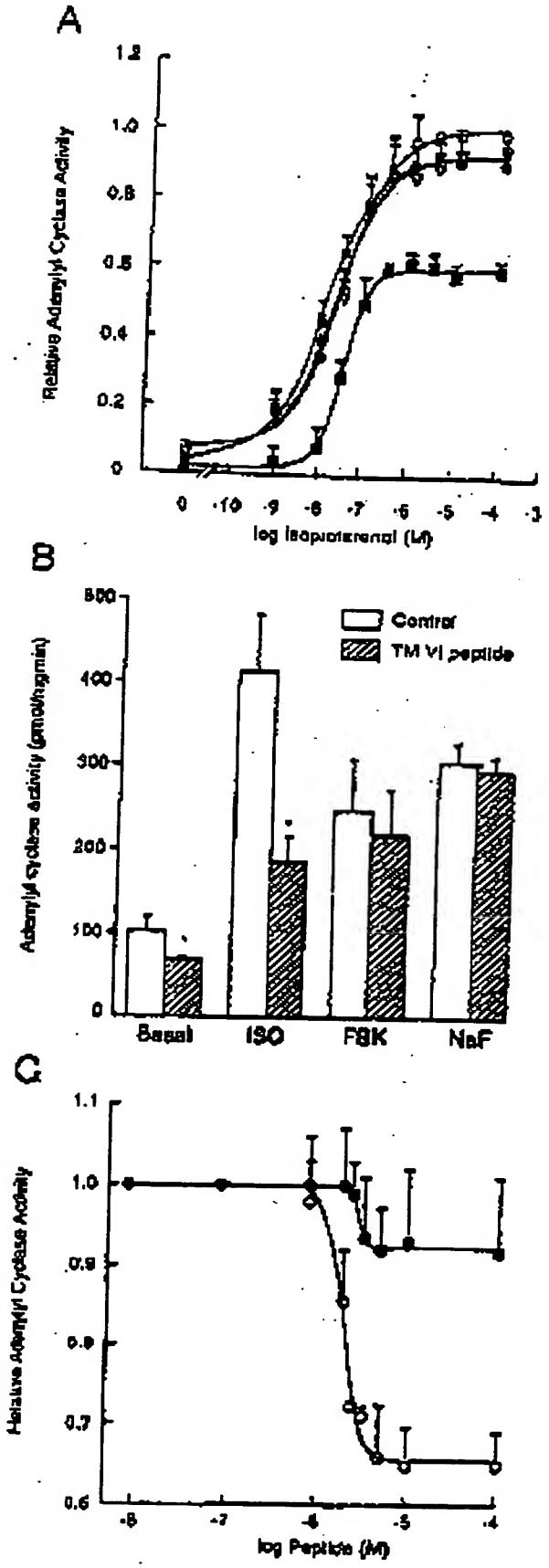

A
Relative Adenylyl Cyclase Activity
log Isoproterenol (M)
B
Adenylyl Cyclase Activity (pmol/mg/min)
Control
TM VI peptide
Basal
ISO
FSK
NaF
C
Relative Adenylyl Cyclase Activity
log Peptide (M)

FIGURE 6

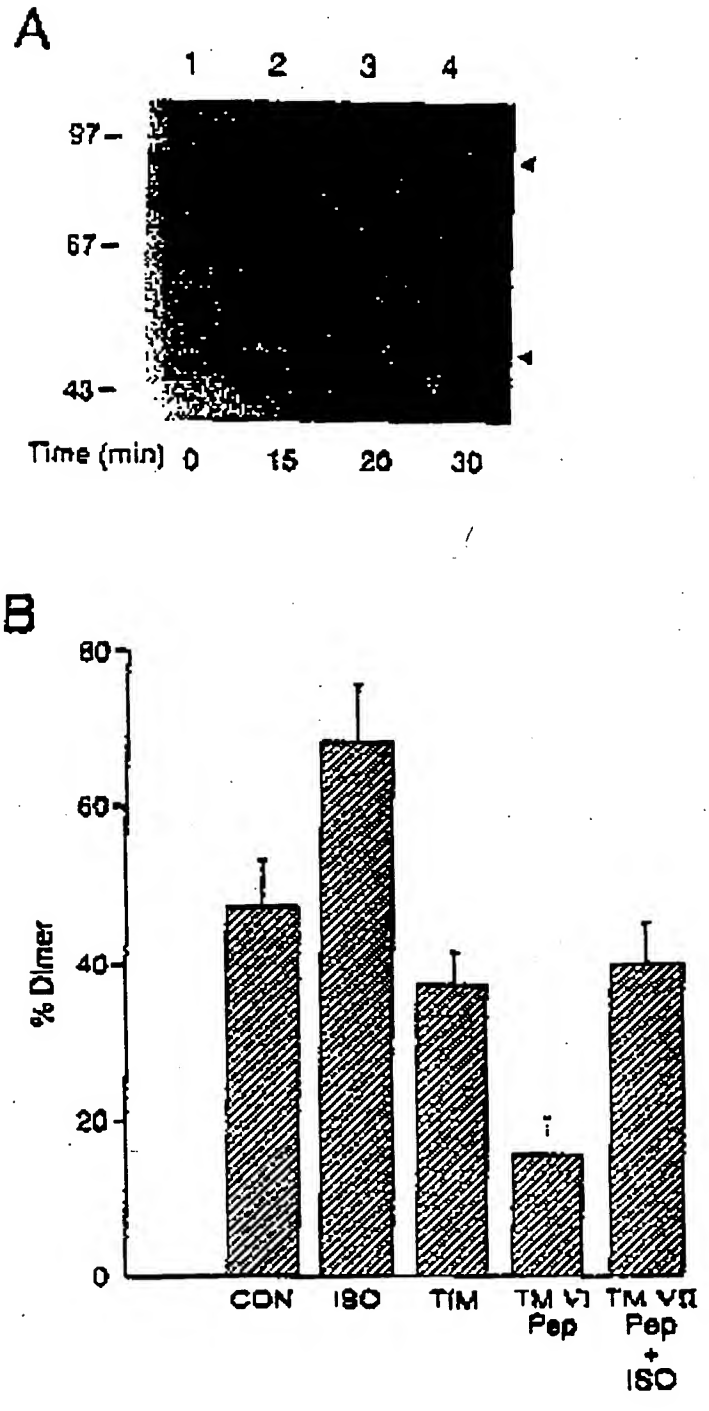
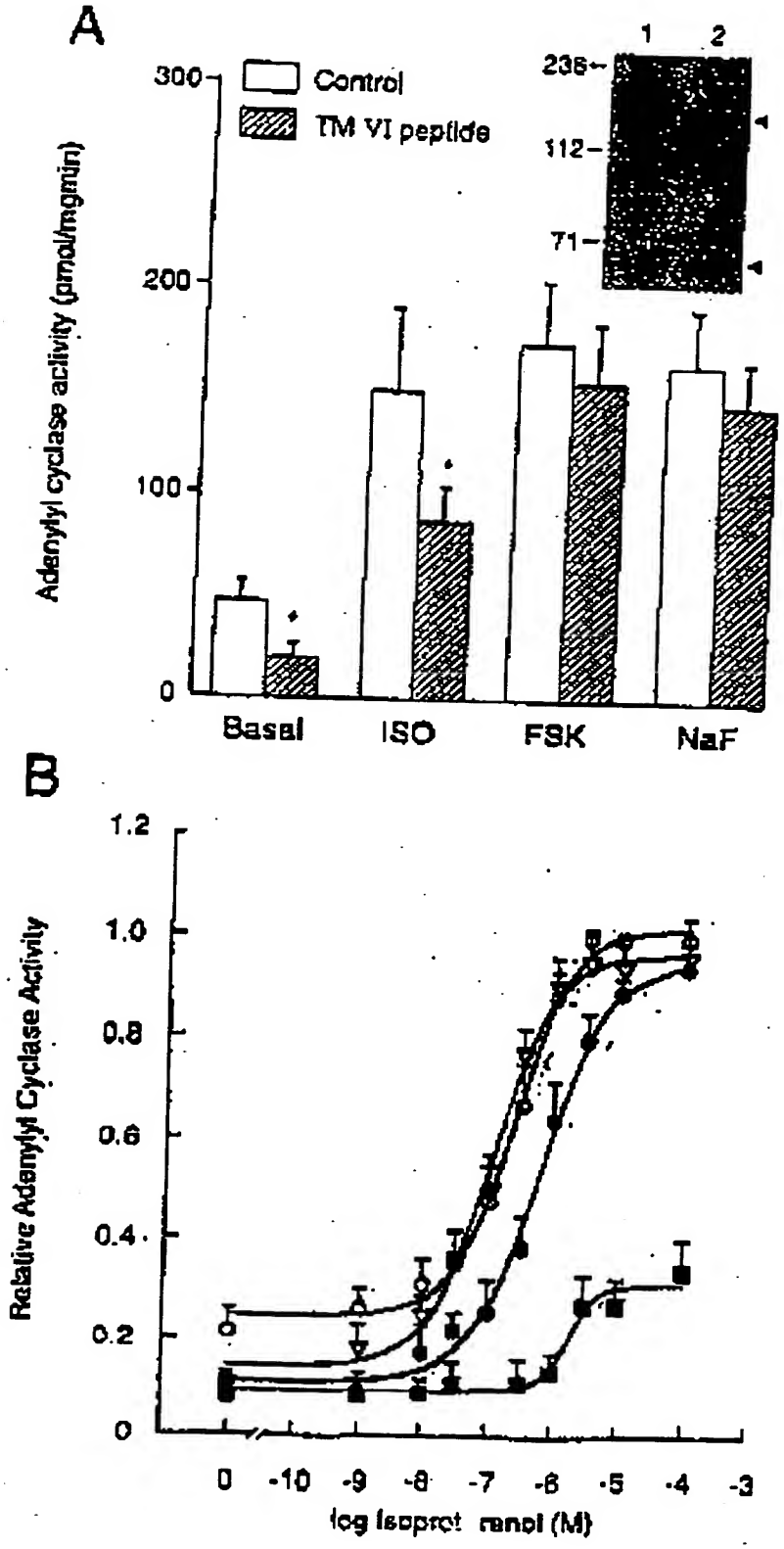

A
1
2
3
4
97-
67-
43-
Time (min) 0
15
20
30
B
80
60
40
20
0
% Dimer
CON
ISO
TIM
TM VI Pep
TM VII Pep + ISO

FIGURE 7



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	G	G	L
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α1B	+	+	+
α1C	+	+	+
α2A	A	+	V
α2B	A	+	+
α2C	A	+	+
β1	+	+	+
β2	+	+	+
β3	+	+	+
Dopamine receptors			
D1	-	+	+
D2	-	-	-
D4C	-	-	-
D5	-	+	+
Vasopressin receptors			
V2	-	-	-
Muscarinic receptors			
M1	-	-	-
M2	-	-	-
M3	-	-	-
M4	-	-	-
M5	-	-	-

Serotonin receptors			
HT1b	+	+	V
HT1c	+	P	+
HT1d	+	+	I
HT1e	+	+	+
HT1f	+	+	I
HT2	+	P	I
HT7	+	+	V
Thymotropin receptor	-	-	-

1) Intra- and Intermolecular interactions in GPCRs studied using peptides or mini-genes

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Lanier et al., (1993) Science 259:1453- same group, same subject

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Wade et al., (1994) Mol. Pharmacol. 45:1191 Multisite interactions of receptors and G proteins: enhanced potency of chimeric receptor peptides in modifying G protein function.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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A3

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(30) Priority Data:
60/021,031 1 July 1996 (01.07.96) US

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BOUVIER, Michel [CA/CA]; 2702 Ch. de la Cote Ste-Catherine, Montreal, Quebec H3T 1B7 (CA). DENNIS, Michael [CA/CA]; 4140 Hampton Avenue, Montreal, Quebec H4A 2K9 (CA). HEBERT, Terence, E. [CA/CA]; 5000 Belanger Street, Montreal, Quebec H1T 1C8 (CA).

(74) Agent: MBM & CO.; P.O. Box 809, Station D, Ottawa, Ontario K1P 5P9 (CA).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
7 May 1998 (07.05.98)

(54) Title: PEPTIDES AND PEPTIDOMIMETIC COMPOUNDS AFFECTING THE ACTIVITY OF G-PROTEIN-COUPLED RECEPTORS BY ALTERING RECEPTOR OLIGOMERIZATION

(57) Abstract

This invention relates to peptides and peptidomimetic compounds that modulate the function of G-protein-coupled receptors by affecting the ratio of receptor monomer to homo-oligomeric forms. Novel short peptides of a preferred length of up to about 15-20 amino acid residues are modeled on transmembrane domains of G-protein-coupled receptors, whose activities are affected by the formation of oligomers. These novel peptides and peptidomimetic compounds can be used to selectively affect the activity of G-protein-coupling receptors, thereby functioning as potential therapeutic drugs, etc.. A preferred peptide is GIIMGTFTLCWLPFFIVNIV.

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INTERNATIONAL SEARCH REPORT

Inte[illegible]al Application No
PCT/IB 97/00814

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C07K7/08 A61K38/10 A61K38/17
G01N33/68 C07K14/72 C07K14/715

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 05695 A (UNIV NEW YORK) 17 March 1994 see page 13, line 29 - page 14, line 39 see page 20, line 28 - page 24, line 23; example 1 ---	1-8, 10-20
X	WO 94 24162 A (VETIGEN ;VIRBAC (FR); LENZEN GERLINDA (FR); KAPOOR ARCHANA (US)) 27 October 1994 see the whole document --- -/--	1-3,7, 10-14,20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
3 March 1998	13. 03. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HEBERT T E ET AL: "A PEPTIDE DERIVED FROM A BETA2-ADRENERGIC RECEPTOR TRANSMEMBRANE DOMAIN INHIBITS BOTH RECEPTOR DIMERIZATION AND ACTIVATION" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 27, 5 July 1996, pages 16384-16392, XP002045155 cited in the application see the whole document	1,2,4,5, 7-14,19, 20
E	WO 97 35881 A (NG GORDON Y K ;SEEMAN PHILIP (CA); GEORGE SUSAN R (CA); DOWD BRIAN) 2 October 1997 see the whole document	1-18,20
E	WO 97 40148 A (UNIV MONTREAL L ;BOUVIER MICHEL (CA); LOISEL THOMAS P (CA); HEBERT) 30 October 1997 see the whole document, especially page 12, lines 9-17	1-20
A	GREGORY, H. ET AL.: "Luteinizing hormone release from dissociated pituitary cells by dimerization of occupied LHRH receptors." NATURE, vol. 300, 18 November 1982, pages 269-271, XP002057464 see abstract see page 271, right-hand column, line 15 - line 20	1-20
A	WO 90 08160 A (IMP CANCER RES TECH) 26 July 1990 see page 9, paragraph 2; claim 1	1-18,20
A	WAGNER T ET AL: "DIFFERENTIAL REGULATION OF G-PROTEIN ALPHA-SUBUNIT GTPASE ACTIVITY BY PEPTIDES DERIVED FROM THE THIRD CYTOPLASMIC LOOP OF THE ALPHA2-ADRENERGIC RECEPTOR" FEBS LETTERS, vol. 365, no. 1, 1995, pages 13-17, XP002045148 see abstract; table 1 see page 16, left-hand column, paragraph 3 - right-hand column, paragraph 1	1-20

1

INTERNATIONAL SEARCH REPORT

Int. application No.
PCT/IB 97/00814

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 8 and 15-18 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 97/00814

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9405695 A	17-03-94	AU 4855393 A	29-03-94
		US 5508384 A	16-04-96
WO 9424162 A	27-10-94	FR 2704235 A	28-10-94
		AU 6573094 A	08-11-94
		US 5656440 A	12-08-97
WO 9735881 A	02-10-97	AU 2020497 A	17-10-97
WO 9740148 A	30-10-97	AU 2501297 A	12-11-97
WO 9008160 A	26-07-90	NONE	